ISOLATION AND EXPANSION OF A UNIQUE STEM CELL POPULATION FROM HUMAN AND MOUSE GALLBLADDERS

by

Rohan Raoul Manohar

Biology B.S., Denison University, 2006

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School of Medicine

This dissertation was presented

by

Rohan R Manohar

It was defended on

April 12th, 2012

and approved by

George Michalopoulos, MD PhD, Department of Pathology

Stephen Strom, PhD, Department of Pathology

Ira Fox, PhD, Department of Surgery

Anthony Demetris, MD, Department of Pathology

Kyle Orwig, PhD, Department of Obstetrics, Gynecology and Reproductive Sciences

Thesis Advisor: Eric Lagasse, PharmD PhD, Department of Pathology

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The identification of resident stem cells in the gallbladder and liver has great importance for basic biology and cell-based therapy. However, there is a paucity of data regarding gallbladder stem cells, especially in humans. In addition, the isolation of liver stem cells has been controversial in particular their ability to robustly generate hepatocytes that can engraft *in vivo*. The differences between gallbladder cells that are part of the extrahepatic bile duct system and intrahepatic bile duct cells have not yet been explored. Finally, the identification of gallbladder stem cells could represent an alternative source of hepatocytes for cell-based therapy to cure end stage liver disease. This is because gallbladder and liver cells are developmentally related and the removal of adult human gallbladders is relatively common surgery, thereby providing a large source of donor tissue.

In this study, we focus on developing *in vitro* expansion and differentiation assays to evaluate bile duct stem cells, but focus on the adult mouse and fetal human gallbladders. We were able to successfully isolate and expand a resident epithelial stem cell from adult mouse gallbladder and intrahepatic bile duct system. Furthermore, we show that these cells are distinct or unique from each other. In addition, we have identified cell surface markers that can be used for the prospective isolation of candidate fetal human gallbladder stem cells and have determined that these cells have distinct phenotypic profiles compared to intrahepatic bile duct cells. Last, we identified candidate transcription factors that can be used for the reprogramming of

gallbladder stem cells into hepatocytes. These experiments set the stage for future studies that could have important ramifications for cell-based therapy for liver disease.

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LIST OF ABBREVIATIONS

IHBD: Intrahepatic bile duct cell

EHBD: Extrahepatic bile duct cell

CBD: Common bile duct

Embryonic Day: ED

Hes1: Hairy and Enhancer of split homolog-1

Pdx1: Pancreatic and Duodenal homeobox-1

SOX: Sry (Sex determining region)-box

BDL: Bile Duct Ligation

CCl4: Carbon Tetrachloride

Gallbladder Cancer: GBC

CD: Cluster of Differentiation

XX

OLT: Orthotopic Liver Transplantation

EGF: Epidermal growth factor

HGF: Hepatocyte growth factor

Dex: Dexamethasone

HNF4α: hepatocyte nuclear factor alpha

NOD/SCID: non-obese diabetic/ severe combined immunodeficiency

FOXA3: Forkhead box A3

iHep: induced hepatocyte-like

FAH: Fumarylacetoacetate Hydolase

NTBC: 2-(2-nitro-4-trifluoro-methylbenzyol)-1,3-cyclohexanedione

AFP: α-fetoprotein

TGFα: transforming growth factor alpha

bFGF: basic fibroblast growth factor

FACS: Fluorescence activating cell sorting

MACS: Magnetic activating cell sorting

TEM: Transmission Electron Microscopy

MDR: Multidrug resistance

SAM: Significance Analysis of Microarray

IFN: Interferon

AAT: Alpha-1-antitrypsin.

iPS: induced pluripotent stem cells

C/EBPα: CCAAT-enhancer binding protein alpha

AQP: Aquaporins

PREFACE

One chapter of this dissertation contains a peer-reviewed published manuscript on which I am first author:

Manohar R, Komori J, Guzik L, Stolz DB, Chandran U, LaFramboise W, Lagasse E. Identification and expansion of a unique stem cell population from adult mouse gallbladder. Hepatology. *2*011 Nov; 54(5): 1830-41. Pubmed PMID: 21793026.

1.0 INTRODUCTION

1.1 ANATOMY AND FUNCTION OF THE BILE DUCT SYSTEM

The bile duct system is divided into two parts: the intra- and extrahepatic bile duct systems. The intrahepatic bile duct (IHBD) cells are epithelial cells that line the luminal spaces –bile ducts- within the liver through which bile is transported from the liver to the extrahepatic bile ducts (EHBDs) (1). The EHBD system consists of the common hepatic duct, gallbladder, cystic duct and common bile duct (CBD) (2, 3). Bile is concentrated and stored in the gallbladder, from where it is pumped through the CBD into the duodenum (4, 5). The gallbladder therefore plays an important role in the digestive process. However, this role appears to be dispensable, as cholecystectomy or removal of the gallbladder, does not have major long-term consequences on the health of the organism. This apparently non-essential function of the gallbladder has been discussed as early as 1922, when McMaster determined that rats, horses, doves and deer among other animals do not possess gallbladders (6). Till today the complete functions of the gallbladder and why animals without one live normal lives, are not well understood. The bile duct system itself was formerly thought to be a mere conduit of bile. However, it has recently become clear that bile duct cells have important secretory functions and modify the content of bile (7), and might also play a role in mucosal immunity (8). In all, the basic biology of bile duct cells is not well understood. In this study, we focus on resident stem cells in the IHBD and

EHBD systems, compare them to each other, and evaluate the potential therapeutic significance of gallbladder stem cells, in order to better elucidate bile duct biology.

1.2 DEVELOPMENT OF THE BILE DUCT SYSTEM

The liver, EHBD system and the ventral pancreas develop from the posterior ventral foregut endoderm (9-11). The liver primordium is formed by the closure of the lateral and medial endodermal domain of foregut (11), followed by its evagination into the septum transversum, which is the mesenchymal tissue located between the cardiac mesoderm and the ventral endoderm (10). The ventral pancreas is induced in the lateral endodermal cells that are caudal to the hepatic endoderm. Both these events occur under the inductive effects of neighboring mesenchymal cells (10) at embryonic day (ED) nine and three weeks in the mouse and human respectively.

1.2.1 Developmental link between the EHBD system and ventral pancreas

The cranial portion of the liver primordium after its evagination into the septum transversum gives rise to the liver including the IHBD cells, while the caudal portion is thought to give rise to the EHBD system (9, 12). However, this notion is based on histological data that show that EHBD and IHBD systems develop together. The exact mechanism of development and the cell-intrinsic factors by which the IHBD and EHBD segregate have heretofore been unclear.

Sumazaki et al. (13) have noted gallbladder agenesis and ectopic pancreatic tissue in the CBD in Hairy and Enhancer of split homology-1 (*Hes1*) deficient mice. Fukuda et al. (14)

corroborated these data in a later study where they observed ectopic pancreatic tissue in the CBD of *Hes1*-knockout mice. To an extent, these data are not surprising as HES-1 is a downstream effector in the Notch signaling cascade, and the Notch pathway is well characterized as being important for bile duct development (15, 16). What is interesting about these studies is the apparent difference in IHBD and EHBD ontogeny. In both reports, IHBD system development appeared normal though the connection to the CBD was lacking (13, 14). Moreover, the authors did not observe pancreatic cells in the developing IHBD, suggesting a shared ontogeny between the EHBD and the ventral pancreas. This possibility is further supported by reports of congenital defects in humans wherein both the pancreas and EHBD system are affected (17-19).

1.2.2 IHBD and EHBD systems develop from separate progenitors

This shared ontogeny between the EHBD system and ventral pancreas has been much elucidated in a recent study by Spence et al. (20). The authors used a Pancreatic and Duodenal homeobox-1 (PDX1)-Cre mouse to demonstrate that hepatocytes and IHBD cells derive from PDX1- cells while EHBD cells and ventral pancreas derive from PDX1+ cells. They found that SRY- (sex determining region)-box (SOX)-17 controlled the specification of the EHBD and pancreatic cells from PDX-1+ cells and this specification required HES-1. SOX-17 loss-of-function at ED 8.5 resulted in gallbladder agenesis and the presence of ectopic pancreatic tissue (PDX-1+ cells) in the EHBD. Conversely SOX17 gain-of-function at ED 9.5 resulted in ectopic ductal tissue (SOX-17+ cells) in the developing pancreas. In both the foregoing conditions, the IHBD system was not affected. The regulation of SOX-17 expression and its interaction with HES-1 and PDX-1 have yet to be completely understood. However, these data definitively

demonstrate that during murine development, the IHBD and EHBD systems originate from distinct progenitor cells.

1.2.3 Developmental Diseases of the bile duct system

The most common developmental defect of the bile duct system is biliary atresia, wherein the bile ducts exhibit improper development resulting in their eventual obstruction and destruction (21-23). Biliary atresia presents in the neonatal period but the etiology remains unclear and the only cure is liver transplantation. Subsequently, it is the most common indicator of pediatric liver transplant (24). Another common developmental disorder is Alagille syndrome, which is autosomal-dominant and primarily characterized by a paucity of bile ducts, resulting in bile accumulation in the liver (23). While it is known that mutations in the Notch ligand Jagged-1 are responsible for the majority of patients with this disease (25, 26), the overall etiology of the disease is unknown. Subsequently, the only cure for Alagille syndrome is liver transplantation. Overall, the characterization of resident stem cells in the IHBD and EHBD systems has great importance for better understanding bile duct developmental disorders, designing therapeutic strategies to eliminate them, and elucidating the differences between IHBD and EHBD cells.

1.3 STEM CELLS IN THE GALLBLADDER

1.3.1 Heterogeneity in the gallbladder epithelium

Understanding of the heterogeneity of parenchymal cells within an organ and how these cells interact during homeostasis is essential to the characterization of the resident stem cells in that organ. The epithelium of the adult mouse and human gallbladders consist of a single layer of columnar epithelial cells (27). In the human gallbladder, Rokitansky-Aschoff (R-A) sinuses, which are invaginations of the epithelial cells through the muscle layer into the subserosal connective tissue have been identified by histology (28). However, R-A sinuses are considered to represent early pathological changes in the gallbladder as their presence has been noted in gallbladder cancer (29, 30) and in gallstones (31, 32). Their existence in the uninjured gallbladder is controversial.

Four types of epithelial cells that have been characterized in the mouse and human gallbladders - Principal cells, Brush cells, Pencil-shaped cells, and Granular cells – of which, Principal cells are the most numerous (33), followed by Brush cells (34, 35). However, the discrimination of each cell type from the others has only been performed by electron microscopy (33, 36) and there are no available markers for their isolation. As a result, their function in the gallbladder and role in tissue homeostasis are not well understood.

1.3.2 Self-renewal of the gallbladder epithelium

The rate of self-renewal in the human gallbladder epithelium during normal homeostasis is very low (37). In addition, autoradiographic studies using 3H-thymidine in guinea pig, mice, hamster

and rabbit gallbladders indicate that self-renewal potential is spread fairly evenly over the entire epithelium (37). Self-renewal potential has also not been linked to a specific epithelial cell type in the gallbladder.

1.3.3 Stem cells in the EHBD system

There are few reports of the identification of stem cells in the EHBD system, and specifically in the gallbladder. Irie et al. (38) observed c-kit+ cells in the mouse CBD and gallbladder following bile duct ligation (BDL) that expressed albumin and cytochrome P450 enzymes. These cells engrafted into livers of mice injured with carbon tetrachloride (CCl_4) and one-third partial hepatectomy, suggesting that they were capable of hepatocyte differentiation. However, this study does not mention the presence c-kit+ cells in the uninjured EHBD, which makes evaluating the origin of these cells difficult.

Recently, there have been reports indicating that peribiliary glands (PBGs) in the bile duct system are a reservoir for multipotent stem cells that are capable of differentiation into hepatocytes, bile duct cells and pancreatic islets (3, 39). PBGs are mucus secreting epithelial cells buried in the bile duct walls and are mostly found in the CBD and the Ampulla of Vater (40-42). They express the transcription factors SOX-9, SOX-17 and PDX-1 (39) and contain cells capable of differentiation into hepatocytes, bile ducts, and glucose-responsive pancreatic islets *in vitro* and *in vivo* (3). However, these reports while interesting, fall short of definitively identifying the initial cell population isolated from the primary tissue. Furthermore, the gallbladder does not contain PBGs (3). Therefore, there is a clear paucity of data for the identification of stem cells in the gallbladder.

1.3.4 Stem cells in gallbladder cancer

Gallbladder cancer (GBC) is a rare cancer but is the most common malignancy of the bile duct system (43). The etiology of this disease is not well understood and the only current clinical treatment for GBC is resection of the organ. Furthermore, even with advancements in surgical technique, the five-year survival rate of patients with advanced GBC is only 10% (44, 45). Similar to the normal gallbladder, there is a dearth of data for identification of stem cells in GBC.

Two recent reports have shed some light on the cell-of-origin in GBC. In 2010 Shi et al. (46) demonstrated that primary human GBC consisted of a discrete Cluster of Differentiation (CD) 44+CD133+ subpopulation of cells that was enriched for spheroid formation *in vitro*, and tumor formation *in vivo*. Interestingly, these cells were also significantly chemoresistant to gemcitabine and 5-fluorouracil, two standard chemotherapeutics. These data were corroborated by another study where the authors found CD133+ cells from primary human GBC to be enriched in spheroid formation, tumor initiation and chemoresistance (47). Therefore, it appears that GBC contains specific tumor-initiating cells, which would need to be targeted therapeutically. However, a thorough understanding of GBC cancer stem cells would not be complete without first elucidating stem cells in the uninjured gallbladder.

1.4 STEM CELLS IN THE IHBD SYSTEM

1.4.1 Heterogeneity of IHBD cells

The liver is composed of two major parenchymal cell types: hepatocytes and IHBD cells. The bile ducts begin with the Canal of Hering within the hepatic lobule, and progressively increase in diameter into a system of interlobular, septal, area, segmental and hepatic ducts that eventually join with the EHBD system (1, 48). IHBD cells that line the Canals of Hering are small, cuboidal cells with high nuclear to cytoplasmic ratio (49, 50). By contrast, larger bile ducts are lined by columnar epithelial cells that have low nuclear to cytoplasmic ratios, apical Golgi apparatus and relatively abundant rough endoplasmic reticulum. Beyond morphology, small and large IHBD cells differ by gene expression pattern and response to injury such as BDL (51, 52). These data have led to the notion that small IHBD cells may represent committed bile duct progenitor cells that give rise to the large IHBD cells upon injury (50).

1.4.2 Self-renewal of IHBD cells

The rate of self-renewal of IHBD cells in the normal uninjured liver is known to be very low (50). Autoradiographic studies using 3H-thymidine in rats have shown that the label retaining cells *in situ* is <1% (53, 54) suggesting that the rate of self-renewal is very low. Furthermore, it is generally believed that during homeostasis in the uninjured liver, new hepatocytes and IHBD cells are generated by duplication of each respective epithelial cell type (55-58). However, in a recent study Furuyama et al. (59) using SOX9 transgenic mouse, which labels most of if not all IHBD cells, demonstrated that new hepatocytes derive from IHBD cells. These data were

contradicted in another more recent paper that used an adenoviral labeling system in the adult mouse to specifically labels hepatocytes. Malato et al. (60) determined that new hepatocytes derive from hepatocytes. Both these studies use state-of-the art tools such as transgenic mice and viral serotypes that afford good target cell specificity and low toxicity, and provide direct evidence for their respective conclusions. Yet the stark difference in these conclusions highlights much of the disagreement surrounding our current knowledge of liver homeostasis, which extends to studies of liver stem cells.

1.4.3 Stem cells in the IHBD system

The presence of a stem cell distinct from IHBD cells and hepatocytes in the uninjured liver is hotly debated because hepatocytes themselves respond to tissue injury such as two-thirds partial hepatectomy (58). It is thought that stem cells do not have a significant role to play in tissue repair and maintenance unless this foregoing ability of hepatocytes is blocked, and most studies involving liver stem cells focus on such liver injury models. Using these models, the Canals of Hering have been identified as a potential stem cell niche as long ago as 1958 (61). In a much more recent report, Kuwahara et al. (62) use a label retention assay to identify the regenerative cells during liver injury. They find that all the candidate stem cell niches localize to or around the Canals of Hering. However, correlating these data to the uninjured liver and the subsequent identification of stem cells has proved difficult.

There are a few studies of the prospective identification of stem cells in the uninjured adult mouse and human livers (63-73). Suzuki et al. (69) and Qiu et al. (70) used CD133 and CD24 expression respectively, to isolate candidate stem cells from normal adult mouse livers. In both reports, the isolated cell populations expanded *in vitro* and appeared to differentiate into

hepatocytes. More recently, Dorrell et al. (72) used specific antibodies developed in house (74) along with CD133 expression, to identify a subpopulation of non-hepatocyte small liver cells capable of *in vitro* expansion and hepatocyte differentiation.

Schmelzer et al. (63, 66) used EpCAM, CD56, CD133 and CD44 expression to separate candidate liver stem cells from both fetal and adult human livers. The authors report that these cells exhibit weak albumin expression, are negative for α-fetoprotein (AFP) and are able to generate hepatocytes *in vivo*. Remarkably, the number of these stem cells stayed constant from development into adulthood. In a later study, the authors found that these stem cells were also highly enriched for telomerase expression adding an additional marker that can be used for their isolation (73). While interesting, these studies suffer from the same general problems. The specific markers used usually co-stain with IHBD cells in the liver confusing interpretation of the results. Expansion of the stem cells was not completely homogeneous but contained contaminating mesenchymal cells, making the discernment of a resident epithelial stem cell difficult. Finally, the hepatocyte differentiation observed with these cells was inconclusive and occurred at very low frequencies. This could be for various reasons, beginning with the lack of a definitive *in vitro* hepatocyte differentiation protocol. However, it could also be because these candidate stem cells are not capable of *de novo* hepatocyte differentiation. In all, the identification of a liver stem cell is inconclusive and consequently, there are no specific markers for its isolation.

1.5 BILE DUCT STEM CELLS AS CELL-BASED THERAPY FOR LIVER DISEASE

Liver disease affects a large part of western society and is among the top causes of death in the US (75). Roughly 10% of all Americans suffer from liver disease. In addition, 170 million people worldwide and 4 million in the US are infected with Hepatitis C (76), which is a major cause of liver failure in the US (77). Currently the only widespread treatment for chronic liver disease is orthotopic liver transplantation (OLT). However, there are over 17,000 people on the waiting liver for a transplant and only 5,000 available donors (78). In addition, OLT is an expensive procedure that not everyone can afford. One alternative to OLT is hepatocyte transplantation, which has had some clinical success (79), but is limited by a shortage of donor livers and by the inability of hepatocytes to expand *in vitro*. For these reasons, alternative cell-based therapies are being pursued to treat liver disease. One such alternative is a liver stem cell. However, as described before the identification of these cells has been controversial. In addition, their differentiation into hepatocytes *in vivo* has not been definitively proven. Last, availability of donor liver stem cells could be hampered by similar limitations that hamper the availability of donor hepatocytes. This problem of availability can be circumvented by the use of a more readily available tissue stem cell such as a gallbladder stem cell.

The gallbladder is considered to be a non-vital organ and is removed during surgery. Since laparoscopic cholecystectomy became clinically available in 1989, the removal of the gallbladder has become routine with 500,000 – 600,000 surgeries every year (80). This sheer volume of available tissue therefore presents exciting opportunities for candidate cell-based therapy.

1.5.1 Differentiation of mouse gallbladder cells into hepatocytes

Mouse gallbladder epithelial cells have been shown to differentiate into hepatocyte-like cells *in vitro* (81). In this study, the authors cultured mouse gallbladder cells in transwell inserts above a layer of myofibroblast feeder cells, a technique that supports expansion of epithelial cells (82). Following *in vitro* expansion, gallbladder epithelial cells were grown between collagen and matrigel in the presence of epidermal growth factor (EGF), hepatocyte growth factor (HGF) and dexamethasone (Dex). In these conditions, the authors noted the upregulation of the hepatocyte markers, hepatocyte nuclear factor alpha (HNF4α) and albumin. The functionality of these hepatocyte-like cells was determined by bile acid synthesis, uptake of low-density lipoprotein and benzodiazepine metabolism (81). This study established a proof-of-concept for the differentiation of gallbladder cells into hepatocytes.

In a later study, the same group observed engraftment of freshly isolated and *in vitro* expanded gallbladder cells in the livers of non-obese diabetic (NOD)/ severe combined immunodeficiency (SCID) mice (83). However, this engraftment was possible only with tremendous injury to the recipient liver. Even then reported engraftment was only 1.94% ± 2.18%, with the majority of donor cells engrafting in the IHBD system (83). Therefore, the *de novo* differentiation of gallbladder cells into hepatocytes does not appear to be very frequent or robust.

1.5.2 Directed differentiation of somatic cells into hepatocytes

Two recent studies in 2011 have detailed the reprogramming of mouse fibroblasts into hepatocyte-like cells using specific transcription factors (84, 85). In both reports, the authors

began by over-expressing transcription factors that play a role in liver development, in mouse fibroblasts and eliminated those that did not give rise to a hepatic phenotype (10, 86, 87). In the first study, Huang et al. (84) observed that over-expression of GATA4, HNF1α and Forkhead box A3 (FOXA3) in *p19*^{Arf}-null (p19^{Arf} is a cell cycle inhibitor) adult mouse fibroblasts, converted them into induced hepatocyte-like (iHep) cells. iHep cells exhibited hepatic morphology and expressed hepatic genes. Moreover, they were able to significantly (~80%) engraft and rescue five out of twelve fumarylacetoacetate-hydolase deficient (*Fah*$^{-/-}$) mice. *Fah*$^{-/-}$ mice are defective in tyrosine metabolism and require the drug 2-(2-nitro-4-trifluoro-methylbenzyol)-1,3-cyclohexanedione (NTBC) to survive (88, 89). Removal of NTBC results in progressive liver failure and the *Fah*$^{-/-}$ mouse represents a model of inducible liver failure.

In the second study, Sekiya et al. (85) observed that over-expression of combinations of two transcription factors –HNF4α and either FOXA1, FOXA2 or FOXA3- in embryonic and adult mouse fibroblasts resulted in iHep cells. Similar to the previous study, the authors found that the iHep cells resembled hepatocytes in morphology and gene expression patterns and rescued significant (40%) numbers of transplanted *Fah*$^{-/-}$ mice. Both these studies confirm that somatic cells can be successfully reprogrammed into functional hepatocytes capable of engraftment *in vivo*. Furthermore, they suggest that reprogramming is more effective with specific transcription factors than defined culture conditions.

1.6 TECHNIQUES TO EVALUATE BILE DUCT STEM CELLS

Stem cells are typically defined as undifferentiated cells that can self-renew at the single cell level and form lineage committed progeny (90, 91). Satisfying this definition within the context

of the bile duct system is difficult for various reasons. First there are no reports that show definitive expansion of bile duct cells from single cells. In addition, lineage commitment of a stem cell is hard without a full understanding of the heterogeneity of epithelial cells in the native organ as is the case with the gallbladder. In the uninjured liver, the presence of a population of stem cells distinct from general bile duct cells is controversial because there are no specific cell surface markers to isolate these stem cells; those markers that do exist co-stain with IHBD cells. In addition, given the confusion over normal turnover in the liver and the lack of definitive hepatocyte and bile duct differentiation protocols, the plasticity of liver stem cells or IHBD stem cells is hard to define.

1.6.1 Feeder cells for *in vitro* expansion of bile duct cells

Stem cells are defined by their ability for single cell self-renewal, which can be assessed by *in vitro* expansion given the appropriate culture conditions. Recent reports have employed the STO cell line and primary angioblast cells as feeder cells to expand and characterize liver stem cells (63, 92, 93). In addition, primary human myofibroblast cells have been used as feeders to expanded human (94) and mouse (82) gallbladder cells. However, definitive single-cell-based expansion of these cells was not reported in these studies. Furthermore, many of these feeders do not enrich for epithelial cells. Therefore, *in vitro* expanded cultures were not a pure population of resident liver stem cells or gallbladder epithelial cells complicating downstream studies.

In order to identify feeder cells that would support robust expansion of bile duct epithelial cells, the growth of fetal liver cells was screened on up to 40 commercially available feeder cell lines (personal communication, Dr. Eric Lagasse). Lethally irradiated cells of the rat

mammary tumor cell line LA7 were found to support robust expansion of fetal hepatoblasts and IHBD cells (unpublished data). In 1984, Ehmann et al. (95) used LA7 feeder cells to expand mouse mammary epithelial cells. Since then, these feeder cells have been used to culture porcine (96) and human normal and tumorigenic bladder (97) epithelial cells and more recently in our lab, human colon cancer cells (98). It appears that these cells support expansion of ductal epithelial cells and represent a viable system to evaluate single cell self-renewal.

1.6.2 Colony forming assays to evaluate bile duct stem cells

Stem cells are defined by their ability for self-renewal, i.e. to form colonies. Therefore, the frequency of cells capable of forming colonies is a measure of the frequency of stem and/or progenitor cells. The limiting dilution analysis (LDA) assay gives a statistically relevant estimate of the frequency of colony forming cells (99). The LDA is an assay to quantify the frequency of a subpopulation of cells with a specific biological function. In the evaluation of stem cells, biological activity is typically defined as the ability to form a colony and the LDA serves to quantify stem and progenitor cells. The LDA was key to the isolation of hematopoietic (100) and neural stem cells (101). Therefore, the LDA can be used to separate a subpopulation of cells that forms colonies from one that does not. Cell surface markers that select for the former subpopulation could be termed stem cell markers as they enrich for colony forming ability. This is important for bile duct stem cells, as there are no definitive cell surface markers for IHBD and gallbladder stem cells. The LDA could therefore be used to evaluate candidate bile duct stem cell markers.

1.7 SPECIFIC AIMS

The identification of bile duct stem cells holds great promise for basic biology and cell-based therapy. Resident stem cells in the gallbladder are to date, unexplored. In addition, there are no definitive markers to identify IHBD stem cells. Developmentally, the IHBD and EHBD systems have been shown to arise from separate progenitor cells (20). The characterization of and direct comparison between adult gallbladder and IHBD stem cells would shed light on whether these two cell types remain different postnatally. It would also help elucidate separate functions if any, of IHBD and gallbladder cells.

The identification of bile duct stem cells and specifically gallbladder stem cells could have significant ramifications for the treatment of chronic liver disease. This is based on two premises. First, the gallbladder is a nonessential organ and laparoscopic cholecystectomy is a routine procedure accounting for ~25% of all surgeries and resulting in 500,000 – 600,000 discarded gallbladders every year (80). In addition, the gallbladder and hepatocytes are derived from the ventral foregut endoderm (12). Based on this related ontogeny, we hypothesize that it would be relatively easier to reprogram a gallbladder cell into a hepatocyte, than a fibroblast into a hepatocyte. Furthermore, as stem cells are more amenable to reprogramming than differentiated cells (102), we propose to attempt hepatic reprogramming with gallbladder stem cells.

It was my goal to isolate and characterize resident stem cell populations in the gallbladder and IHBD system and compare them to each other. In addition, I used microarray analysis to identify transcription factors that can be used for the directed differentiation or reprogramming of gallbladder stem cells into hepatocytes. My

hypothesis was that there is a resident stem cell in the gallbladder that is distinct compared to IHBD cells, and can be easily reprogrammed into hepatocytes.

1.7.1 Specific Aim 1: Isolation and expansion of a unique stem cell population from adult mouse gallbladder

Adult mouse gallbladder cells were expanded *in vitro* on lethally irradiated LA7 feeder cells. Using LDA, I identified cell surface markers that enriched for colony forming ability specifically from single cells. I evaluated the ability of the stem cell enriched populations to expand and differentiate *in vitro*. Differentiation was assessed in a novel three-dimensional matrigel based assay. Finally the phenotypes and expression profiles of gallbladder stem cells and IHBD cells were compared by flow cytometry and microarray analysis, respectively.

1.7.2 Specific Aim 2: Isolation and expansion of unipotent IHBD stem cell population from adult mouse liver

Adult mouse IHBD cells were expanded *in vitro* on lethally irradiated LA7 feeder cells. Using LDA, I attempted to identify cell surface markers that enriched for colony forming ability specifically from single cells. Using assays developed for the evaluation of gallbladder stem cells, I evaluated the stem cell properties of single-cell self-renewal and differentiation of expanded IHBD cells. Finally the plasticity of IHBD cells to differentiate into hepatocytes *de novo* was tested in *Fah*$^{-/-}$ mice.

1.7.3 Specific Aim 3: Isolation and expansion of a unique stem cell population from fetal human gallbladder

Fetal human gallbladder cells were expanded *in vitro* on lethally irradiated LA7 feeder cells. Using LDA, I identified cell surface markers that enriched for colony forming ability specifically from single cells. Using assays developed for the evaluation of gallbladder stem cells, I evaluated the stem cell properties of single-cell self-renewal and differentiation of candidate gallbladder stem cells.

1.7.4 Specific Aim 4: Preliminary differentiation of mouse gallbladder stem cells into hepatocytes

In preliminary experiments, the ability of adult mouse gallbladder stem cells (Specific Aim 1) to differentiate into hepatocytes was tested in the culture system developed by Kuver et al. (81). Hepatocyte differentiation was evaluated by reverse transcriptase polymerase chain reaction (RT-PCR) for albumin and a bile duct marker, cytokeratin (CK) 19. This establishes a proof-of-concept for differentiation of gallbladder stem cells into hepatocytes. Microarray experiments with total gallbladder cells, gallbladder stem cells and hepatocytes were then performed in order to determine which transcription factors can be used to reprogram gallbladder stem cells into hepatocytes.

2.0 IDENTIFICATION AND EXPANSION OF A UNIQUE STEM CELL POPULATION FROM ADULT MOUSE GALLBLADDER

2.1 INTRODUCTION

Understanding the resident stem cell populations of the biliary system has great importance for basic biology and biliary diseases. The biliary tree is divided into the intrahepatic and extrahepatic biliary systems. The latter consists of the gallbladder, cystic duct, and the common bile duct (1). The biliary system is a conduit for bile to be transported from the liver to the intestine. The gallbladder in turn stores the bile and regulates its content and concentration, playing an important role in the digestive process (5, 103).

While there has been a lot of recent interest in the liver stem cell field (104) there is still a paucity of data regarding gallbladder stem cells. The biliary system, hepatocytes and ventral pancreas develop from the ventral foregut endoderm (9, 10). Histological evidence suggesting that both intra- and extrahepatic systems originate from the hepatic diverticulum has led to the hypothesis that they descend from the same progenitor cell. However, the cell-intrinsic factors that result in their specification have heretofore been unclear. Recently, it has been shown that the progenitor cells that give rise to each system separate out during development (20). Using a Pdx1-Cre mouse Spence et al. (20) demonstrated that the hepatocytes and IHBD cells derive

from Pdx1- cells while the EHBD cells and ventral pancreas derive from Pdx1+ cells. Sox17 controls the specification of the EHBD and pancreatic cells. Sox17 loss-of-function embryos exhibit gallbladder agenesis and the presence of ectopic pancreatic tissue in the extrahepatic bile duct. Conversely Sox17 gain-of-function results in ectopic ductal tissue in the developing pancreas. In both cases, the intrahepatic system is not affected. It appears that the IHBD and EHBD cells descend from separate progenitor cells governed by separate transcriptional cascades. It is therefore possible that adult IHBD and EHBD cells could be distinct as well.

The aims of this study were to isolate and characterize stem cells from the adult mouse gallbladder and compare their phenotypic and expression profiles with IHBD cells. In addition to basic biology, an understanding of gallbladder stem cells would be vital to the study of gallbladder carcinoma, a rare but poorly understand malignancy (43) and congenital diseases involving biliary dysmorphogenesis, such as biliary atresia (21). It would also elucidate the ontogeny of cells in the biliary system.

Stem cells are defined as undifferentiated cells that can self-renew at the single cell level and form lineage committed progeny (91). In this study, we use colony forming and single cell assays along with a morphogenesis assay to characterize an EpCAM+CD49f^{hi} epithelial subpopulation within primary mouse gallbladder that has stem cell characteristics. The gallbladder stem cells can be propagated *in vitro* through long-term passage (>passage 20) and can engraft in the subcutaneous space of recipient mice. Last the gallbladder stem cells and IHBD cells have distinct expression profiles. These data represent one of the first reports to isolate and characterize the resident stem cell population in the adult mouse gallbladder.

2.2 MATERIAL AND METHODS

2.2.1 Gallbladder cell isolation and culture

Gallbladder cells were isolated from C57BL/6-Tg (UBC-GFP) 30Scha/J mice (Jackson Laboratory, ME). Gallbladders were removed, cut in half, rinsed in HBSS to remove bile and incubated in EBSSA/10mM EGTA/1% HEPES for 10min. The tissue was treated with 1 mg/ml CollagenaseII (Invitrogen, CA) + 100 μg/ml of DNaseI (Roche, IN) for 1 hour followed by 0.25%Trypsin /0.1%EDTA (Fisher Scientific, MA) for 30 min to obtain a cell suspension. Cells were plated on irradiated feeders at ~8,000 cells/cm^2 and grown in DMEM/F-12 supplemented with 0.5% FBS, 25μg/ml gentamycin (Sigma-Aldrich, MO) and 1% Insulin-Transferrin Selenium (ITS) (Mediatech, Inc, VA). When cultures were ~70% confluent (2-3 weeks post plating), they were passaged by incubation with 10mM EGTA/1% HEPES followed by 0.25% Trypsin /0.1%EDTA (Mediatech Inc., VA).

2.2.2 Preparation of feeder cells

LA7 (ATCC: CRL-2283™) cells were grown in DMEM/F-12 supplemented with 5%FBS, 1% Pen/Strep (Mediatech Inc., VA), 50nM Hydroxycortisone (Sigma-Aldrich, MO) and 5μg/ml Insulin (Sigma-Aldrich, MO). Cells were detached from the dish with 0.25%Trypsin/0.1%EDTA and γ-irradiated at 17,000 rads. Cell culture flasks were seeded at ~70,000 cells/mm^2 to generate a confluent monolayer of feeder cells.

2.2.3 Immunofluorescence

Antibodies against EpCAM, CK19, CD49f and GFP were used at the appropriate dilution in PBS (Table 1). Sections were blocked with 0.5% milk and stained with either anti-rat or anti-rabbit Alexa Fluor® secondaries (Invitrogen, CA). Paraffin sections were stained with EpCAM according to manufacturer's instructions. Images were taken using an IX71 Inverted microscope (Olympus, PA).

Table 1: Comprehensive list of antibodies by flow cytometry and immunohistochemistry/immunofluorescence

Marker	Manufacturer	Dilution
CD2-PE	BD Biosciences	1:50
CD3e-APC	BD Biosciences	1:50
CD4-PE	BD Biosciences	1:50
CD5-PE	BD Biosciences	1:50
CD8a-Biotin	BD Biosciences	1:50
CD9-Biotin	BD Biosciences	1:50
CD11b-APC	BD Biosciences	1:50
CD13-PE	BD Biosciences	1:10
CD14-PE	BD Biosciences	1:10
CD19-PE	BD Biosciences	1:10
CD26-Biotin	AbD Serotec	1:25
CD29-APC	BioLegend	1:50
CD31-APC	BD Biosciences	1:50
CD44-PE	BD Biosciences	1:50
CD45-PE	BD Biosciences	1:10
CD49f Uncon	BD Biosciences	1:100
CD49e-PE	BD Biosciences	1:10
CD49f-APC	BD Biosciences	1:10
CD54-Biotin	BD Biosciences	1:50
CD56-APC	GeneTex Inc	1:50
CD73-PE	BD Biosciences	1:10
CD81-PE	BD Biosciences	1:50
CD86-PE	BD Biosciences	1:10
CD95-PE	BD Biosciences	1:50
CD117-APC	BD Biosciences	1:10
CD121a-PE	BD Biosciences	1:50
CD132-Biotin	BD Biosciences	1:50
CD133-Biotin	eBioscience	1:100
CD166-PE	eBioscience	1:10
CK19	Gift from M. Grompe	1:300
CXCR4-PE	BD Biosciences	1:50
DBA-Biotin	Vector Labs	1:450
Dlk-PE	MBL International	1:50
EpCAM Uncon	AbCAM	1:150
EpCAM Uncon	BD Biosciences	1:50
EpCAM-PE	BioLegend	
EpCAM-APC		
EpCAM-APCCy7		
EpCAM-Biotin		1:25
F4/80-Biotin	Caltag Laboratories	1:50
GFP	AbCAM	1:300
Gr1.1-APC	BD Biosciences	1:50
Sca1-PE	BD Biosciences	1:50

SiglecF F-PE	BD Biosciences	1:50
TER119-PE	BD Biosciences	1:10
Qa1-Biotin	BD Biosciences	1:50

2.2.4 Fluorescence Activated Cell Sorting (FACS) Analysis

Single cell suspensions were stained with appropriate antibodies (Table 2) at 1e6 cells/tube and analyzed on the BD FACSCanto or BD FACSAriaII. Singlet discrimination was performed as described by Wersto et al. (105). Post-acquisition analysis was carried out in FlowJo (http://www.treestar.com). Limiting Dilution Analyses (106) were performed by sorting 1, 5, 10, 15, 25, 50, 100 and 500 cells/well into respective rows of 96-well plates (Corning, NY) seeded with irradiated feeders. Colonies were scored after 3-4 weeks post-plating and candidate stem cell frequencies of sorted subpopulations determined in L-Calc™ (StemCell Technologies, Vancouver). χ^2 statistics for group differences was calculated by ELDA (http://bioinf.wehi.edu.au/software/elda/). Index Sorts were performed in CellQuestPro (BD Biosciences, CA) by sorting single cells into 96-well plates. In clonogenic assays, single cells were sorted into 384-well plates (Nalge Nunc International, NY) seeded with LA7 feeders (Sort Precision Mode: Y0P32Ph4S).

Table 2: Phenotypic profile of gallbladder cells and IHBD cells

Primary, expanded and clonal gallbladder (GB) cells and primary IHBD cells were screened against the 38 antibodies in the panel. For primary GB and IHBD cells, primary cell isolates were co-stained with EpCAM to identify epithelial cells. For expanded cells, GFP expression was used to gate out feeder cells. Representative data for clones B21 and clones N12. +: positive expression; -: negative expression; Het: heterogeneous expression with negative/positive or high/low subpopulations; Low: weak yet distinct staining.

Marker	Primary GB	Primary IHBD	Expanded GB (p6)	Clone B21 (p4)	Clone N12 (p4)
CD2	-	-	-	-	-
CD3e	-	-	-	-	-
CD4	-	-	-	-	-
CD5	-	-	-	-	-
CD8a	-	-	-	-	-
CD9	+	+	+	+	+
CD11b	-	-	-	-	-
CD13	-	-	-	-	-
CD14	-	-	-	-	-
CD19	-	-	-	-	-
CD26	+/Low	+/Het	+/Low	+/Low	+/Low
CD29	+	+	+	+	+
CD31	-	-	-	-	-
CD44	-	-	-	-	-
CD45	-	-	-	-	-
CD49e	-	+	-	-	-
CD49f	Het	+	+	+	+
CD54	-	+	-	-	-
CD56	-	-	-	-	-
CD73	-	+	-	-	-
CD81	-	+	-	-	-
CD86	-		-	-	-
CD95	-	-	-	-	-
CD117	-	-	-	-	-
CD121a	-		-	-	-
CD132	-		-	-	-
CD133	+	+	+	+	+

CD166	+	+	+	+	+
CXCR4	-	-	-	-	-
DBA	+/Het	+/Het	+/Het	+/Het	+/Het
Dlk	-	-	-	-	-
F4/80	-	-	-	-	-
EpCAM		+	+	+	+
Gr1.1	-	-	-	-	-
Sca1	Het	Het	+	+	+
Siglec F	-	-	-	-	-
TER119		-	-	-	-
Qa-1	-	-	+/Low	-	-

2.2.5 Matrigel differentiation Assay

Gallbladder cells were plated in tissue culture dishes at low density (10,000- 15,000 cells/well of 48 well plate) and layered above with an equal volume of Matrigel™ (BD Biosciences, CA) and placed at 37C for 30min. The gels were covered with DMEM/F12 and 1% ITS. Media was changed twice a week. Cysts were isolated by removing the matrigel and incubation with 0.2% Dispase (Invitrogen, CA) and 0.1% CollagenaseII. Single cells suspensions of cysts and ductular structures were prepared with EGTA incubation for 10 min followed by Trypsin as before.

2.2.6 Transport assay with fluorescent dye

Gallbladder cells were cultured in 96-well IbidiTreat Plates (Applied Biophysics, NY) for ~2 weeks till cysts were visible. Rhodamine 123 and R-(+)-Verapamil addition was carried out as described previously (107). Incubation of fluorescent dye and transport inhibitor were

done at 37C. Images were taken on an FV1000 IX81 Laser Scanning Confocal Microscope (Olympus, PA).

2.2.7 In vivo assay

1e6 of the appropriate cells were resuspended in 100 μl of 1:1 HBSS/Matrigel in injected into the subcutaneous neck region of six to eight week old $Rag2^{-/-}\gamma C^{-/-}$ mice. Engrafted areas were removed and fixed with 2%PFA for 1 hour. Cells were re-isolated by the same protocol as primary gallbladder.

2.2.8 Electron Microscopy

Harvested tissues or cells were plunged fixed in 2.5% gluteraldehyde in phosphate-buffered saline (PBS) and stored at $4^{o}C$ for at least 1 day. Cysts in matrigel were similarly fixed *in situ*, and the matrigel was removed and processed separately. Subsequent processing was performed as described by Stolz et al. (108). Sections were viewed with a JEM 1011 transmission electron microscope (JEOL Peabody, MA) at 80kV.

2.2.9 Isolation of IHBD cells

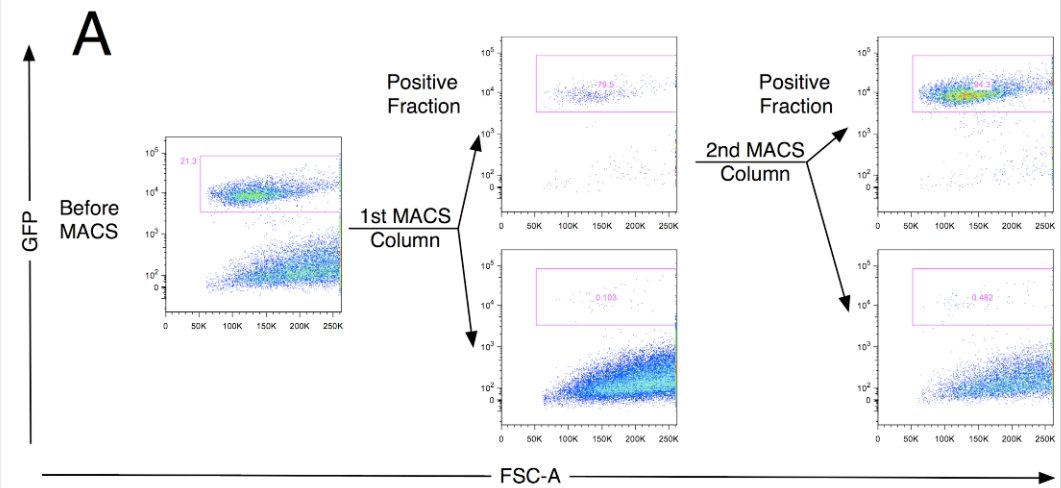
Livers of GFP+ mice were perfused by the 2-step collagenase protocol (109). Low spin fractions (50g for 2 min) were separated from the resulting cell suspension, following which the high spin fraction (400g for 5min) was separated and stained with EpCAM-Biotin (Supplementary Table 2) and streptavidin conjugated microbeads (Miltenyi Biotec, CA) and

eluted through a MS magnetic activated cell sorting (MACS)® separation column (Miltenyi Biotec, CA). The positive fraction was plated on irradiated feeders and grown similar to the gallbladder cells.

2.2.10 Oligonucleotide microarrays

Expanded gallbladder and IHBD cells were stained with EpCAM-Biotin and eluted through two sequential MS MACS® separation columns (Figure 1). RNA was purified by Qiagen miRNeasy Kit (Qiagen, MD). Criteria for sample inclusion in array studies was a purified RNA spectrophotometric absorption ratio 260/280>1.8 (NanoDrop, Wilmington, DE) and RIN value >8.0 via electrophoretic analysis (Agilent Bioanalyzer 2100; Agilent Technologies, CA). In vitro transcription (IVT) performed with 500ng of purified total RNA using the Ambion MessageAmp Premier Enhanced assay protocol (Ambion Inc, TX). cRNA diversity confirmed with the Bioanalyzer 2100 against a Universal Human Reference RNA (Stratagene, CA) to generate an electrophoretogram for each IVT reaction regarding sample yield, integrity, and size diversity. RNA (200 ng) was reverse transcribed followed by 2nd strand synthesis and IVT in the presence of biotinylated nucleotides to produce biotin-11-UTP labeled cRNA. 10.5µg of purified, biotin labeled cRNA was purified, fragmented and hybridized onto Codelink Mouse Whole Genome arrays (Applied Microarrays, AZ) for 18 hours, washed and stained with Alexa Fluor 647 (Invitrogen, CA). Arrays were read using a Genepix® 400B scanner (Axon Instruments, CA) and Codelink Expression Analysis software v5.0 at pixel size 5µm. Final Analysis was performed on raw data normalized to median value for each array followed by combat normalization to remove batch effects (110). Significant transcripts were defined using the Significance Analysis of Microarrays software (v3.0)

(q<0.10) (111). Differentially expressed genes were annotated and analyzed in Ingenuity Pathway Analysis (Ingenuity® Systems CA, www.ingenuity.com). Heatmaps were generated using the open source softwares Cluster (112) and TreeView (http://rana.lbl.gov/EisenSoftware.htm).



B

Population	Purity
GB#1	94.3%
GB#2	91.8%
GB#3	97.4%
GB#4	96.8%
GB#5	96.8%

Population	Purity
IHBD#1	99.4%
IHBD#2	95.5%
IHBD#3	98.7%
IHBD#4	98%
IHBD#5	98.7%

Figure 1 Separation of gallbladder and IHBD cells from feeder cells

(A) Representative flow data showing separation of gallbladder cells from feeder cells with two sequential MACS® separation columns. Similar results were obtained with IHBD cells. (B) Data showing purity of gallbladder and IHBD cells after MACS separation. GB#1-4 represent replicates at different passages *in vitro* of a pool of mouse gallbladder. GB#5 represents a separate pool of mouse gallbladders. IHBD#1-4 represent replicates at different passages *in vitro* from a single mouse liver. IHBD#5 represents a different mouse liver.

2.3 RESULTS

2.3.1 EpCAM is a gallbladder epithelial marker

Gallbladder cells were isolated from GFP donor mice and the epithelial cells separated by flow cytometry. EpCAM, an epithelial surface marker, is expressed on simple epithelial cells such as keratinocytes and thymic epithelial cells (113) as well as on IHBD cells but not hepatocytes, mesenchymal or hematopoietic cells (114). Analysis of mouse gallbladder showed that most epithelial cells are EpCAM+ (Figure 2A). No expression was detected on the mesenchymal cells. To confirm epithelial identity, we performed co-localization studies with EpCAM and CK19, a pan biliary marker (115). Epifluorescence and confocal microscopy performed on acetone-fixed sections show that most CK19+ cells were EpCAM+ (Figure 2B). Therefore, EpCAM marks most gallbladder epithelial cells.

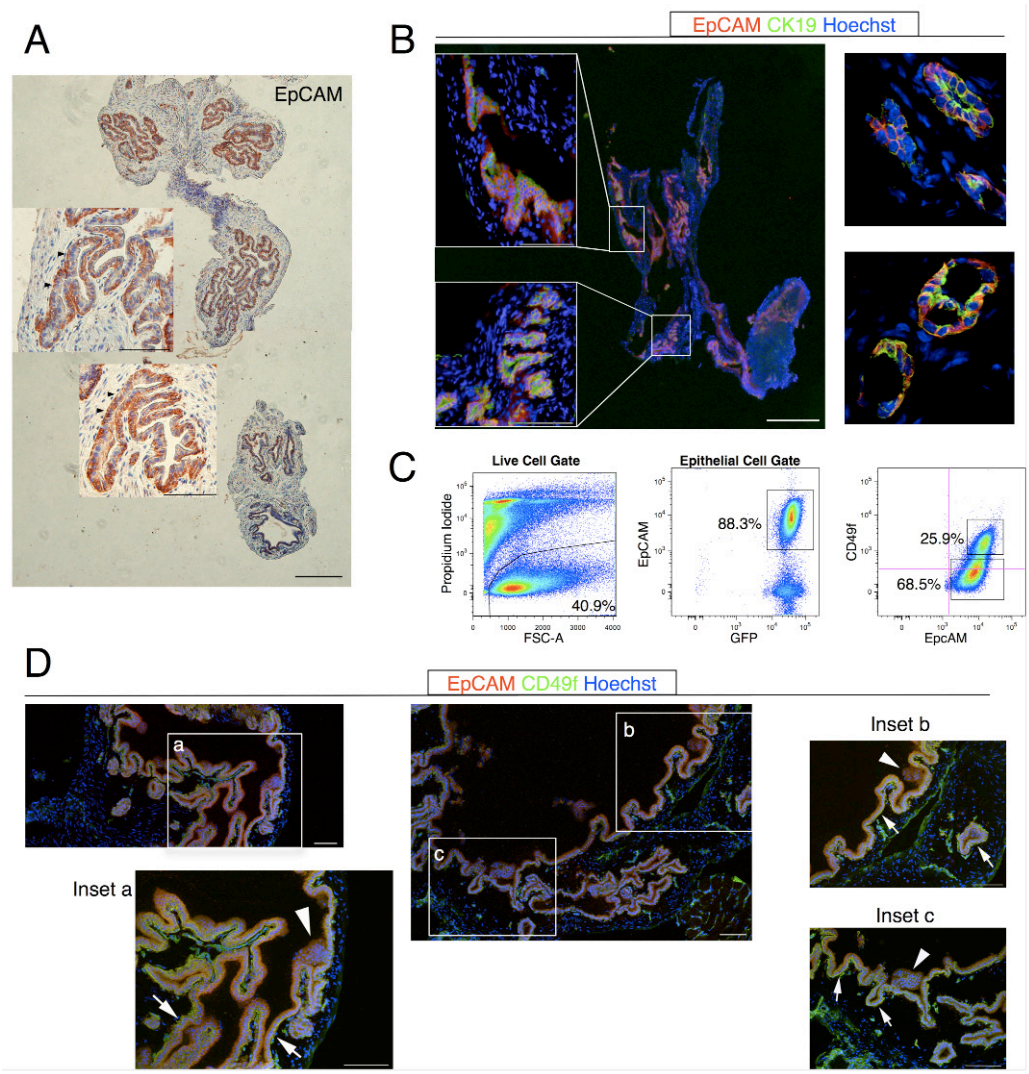


Figure 2 CD49f is heterogeneous in primary gallbladder epithelium

(A) Paraffin sections of mouse gallbladder were stained with EpCAM. EpCAM stained only gallbladder epithelial cells. Basolateral membranes of epithelial cells were EpCAM+ (arrowheads). (B) Acetone-fixed sections were stained with CK19 and EpCAM. Epifluorescent (left panel) and confocal (right panel) micrographs indicate that CK19 co-stains with EpCAM. (C) Flow cytometric analyses on primary gallbladder indicate CD49f heterogeneity (labeled percentages are frequencies of parent population). Crosshairs on the flow plot indicate autofluorescence of unstained cells determined by the control population. (D) Confocal

micrographs of acetone-fixed sections stained with EpCAM and CD49f. White arrows: EpCAM+CD49f^{hi} cells. White arrowheads: EpCAM+CD49f^{lo} cells. Scale bars: 100μm.

2.3.2 CD49f is heterogeneously expressed on Primary Gallbladder epithelial cells

Since there is a paucity of cell surface markers for gallbladder cells, we began screening primary gallbladder for general markers of stem and progenitor cells **(**Table 2). Of the 38 markers we considered, 3 markers - CD49f, DBA and Sca1- were heterogeneously expressed on primary gallbladder epithelial cells (Figure 2C and Figure 3). However, we were only able to separate functionally distinct populations - EpCAM+CD49f^{hi} and EpCAM+CD49f^{lo}- with CD49f. Function in this case is defined by a colony forming assay (see below). Heterogeneous expression of CD49f was confirmed by immunohistochemistry (Figure 2D). Various reports have identified CD49f, integrin α-6, as a stem cell marker in fetal and adult liver (116-118) and other ductal epithelial tissue such as the breast (119, 120). EpCAM+CD49f^{hi} cells expressed markers associated with epithelial stem cells such as CD29, CD133 and Sca1, but not mesenchymal or hematopoietic markers CD31, CD45 and F4/80 (Table 2). These data led us to hypothesize that CD49f is a candidate gallbladder stem cell marker.

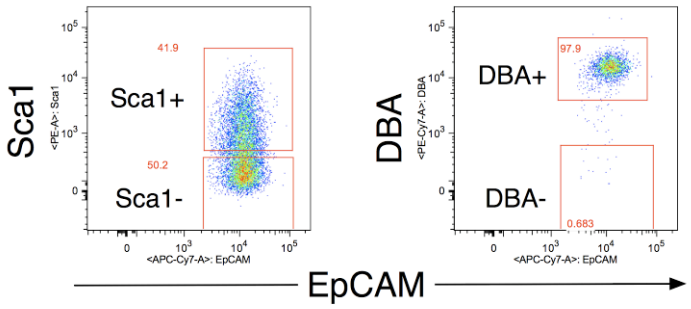


Figure 3 Sca1 and DBA are heterogeneous in primary mouse gallbladder

Sca1 and DBA were screened on primary mouse gallbladder. Debris and cell aggregates were gated out. Sca1 and DBA are heterogeneous in EpCAM+ cells from primary mouse gallbladder.

2.3.3 Gallbladder cells expanded *in vitro* are CD49f+

Gallbladder cells were cultured *in vitro* in conditions that select for epithelial cell growth (95). Briefly, total cell isolate from primary gallbladder was plated on irradiated rat mammary tumor cell line LA7 that served as feeder cells. Transmission Electron Microscopy (TEM) and flow cytometric analyses indicated that there was no fusion between the gallbladder and feeder cells (Figure 4). As stem cells have the capacity for self-renewal, we predicted that expansion *in vitro* would enrich for primitive or stem cells. Flow cytometry analyses of cells after one expansion (p0) showed that only epithelial cells (EpCAM+) expand on the feeders (Figure 5A). EpCAM- cells that were sorted from primary gallbladder did not proliferate (data not shown). Importantly, we found that all gallbladder cells at p0 were CD49f+ (Figure 5B) supporting the notion that *in vitro* expansion selects for EpCAM+CD49f+ primitive epithelial cells.

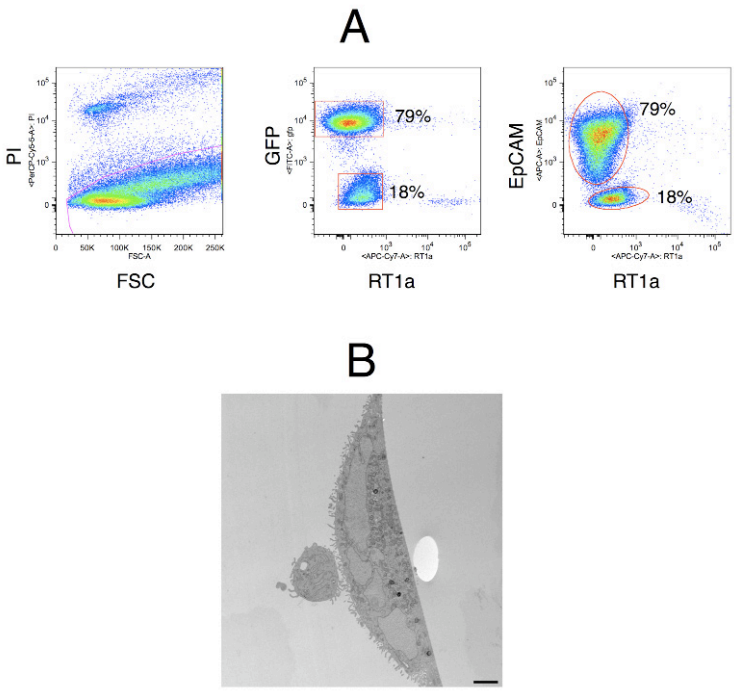


Figure 4 Gallbladder cells and feeder cells do not undergo fusion in vitro

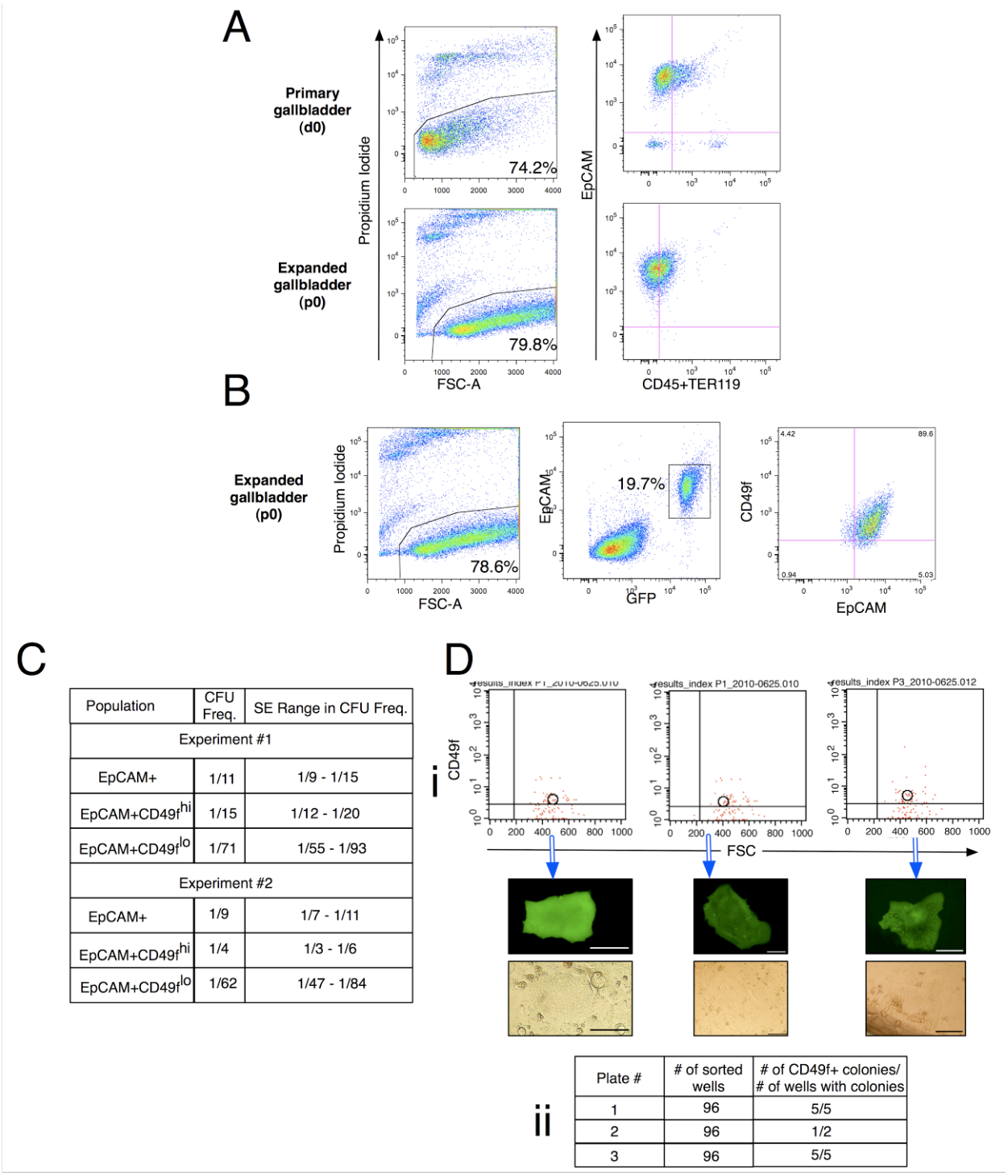
(A) Flow cytometric analyses of gallbladder cells (passage 2). RT1a is the Class I MHC antigen for the rat feeder cells. The middle and right plots are different representations of the live cells in the left plot. The data indicate two populations: GFP+RT1a- and GFP-RTa1+ or EpCAM+RT1a- and EpCAM-RT1a+. (B) Ultrastructual analysis of feeder cell. Feeder cells are large and multi-nucleated indicative of an irradiated cell and are distinct compared to gallbladder cells.

2.3.4 CD49f enriches for gallbladder stem cells

To evaluate CD49f as a gallbladder stem cell marker, we performed LDAs and Index Sorts. The LDA quantifies the frequency of a specific subpopulation of cells with a biological activity (99) and was key to the isolation of hematopoietic (100) and neural (101) stem cells. In the evaluation of stem cells, biological activity is typically defined as the ability to form a colony

and the LDA serves to quantify stem and progenitor cells. We separated EpCAM+CD49f^{hi} and EpCAM+CD49f^{lo} cells from primary gallbladder and performed LDAs. EpCAM+CD49f^{hi} cells exhibited a significantly higher enrichment in colony forming unit (CFU) frequency (ranging from 1/15 to 1/4) compared to EpCAM+CD49f^{lo} cells (1/71 to 1/62) (Figure 5C). χ^2 tests confirmed that the ranges in CFU frequency±SE were significantly different between EpCAM+CD49f^{hi} and EpCAM+CD49f^{lo} cells ($p<0.001$).

We then performed index sorts to confirm these data. An index sort records the phenotype and well number of each deposited cell during a single cell sort. In this manner, the specific surface marker profile of cells that form colonies can be determined retrospectively. In our experiment, 288 single EpCAM+ cells were sorted and the CD49f profile of the each sorted cell was recorded. Retrospective analyses indicated that 11/12 (91.7%) of the colonies that formed originated from CD49f^{hi} cells (Figure 5D). These data together with the LDA results definitively demonstrate that CD49f enriches for candidate gallbladder stem cells.



Population	CFU Freq.	SE Range in CFU Freq.
Experiment #1		
EpCAM+	1/11	1/9 - 1/15
EpCAM+CD49f^{hi}	1/15	1/12 - 1/20
EpCAM+CD49f^{lo}	1/71	1/55 - 1/93
Experiment #2		
EpCAM+	1/9	1/7 - 1/11
EpCAM+CD49f^{hi}	1/4	1/3 - 1/6
EpCAM+CD49f^{lo}	1/62	1/47 - 1/84

Plate #	# of sorted wells	# of CD49f+ colonies/ # of wells with colonies
1	96	5/5
2	96	1/2
3	96	5/5

Figure 5 CD49f expression enriches for gallbladder stem cells

Gallbladders (n≥5) from male and female mice were pooled and analyzed or sorted. (A) Flow cytometric analyses on primary and expanded mouse gallbladder cells show that only

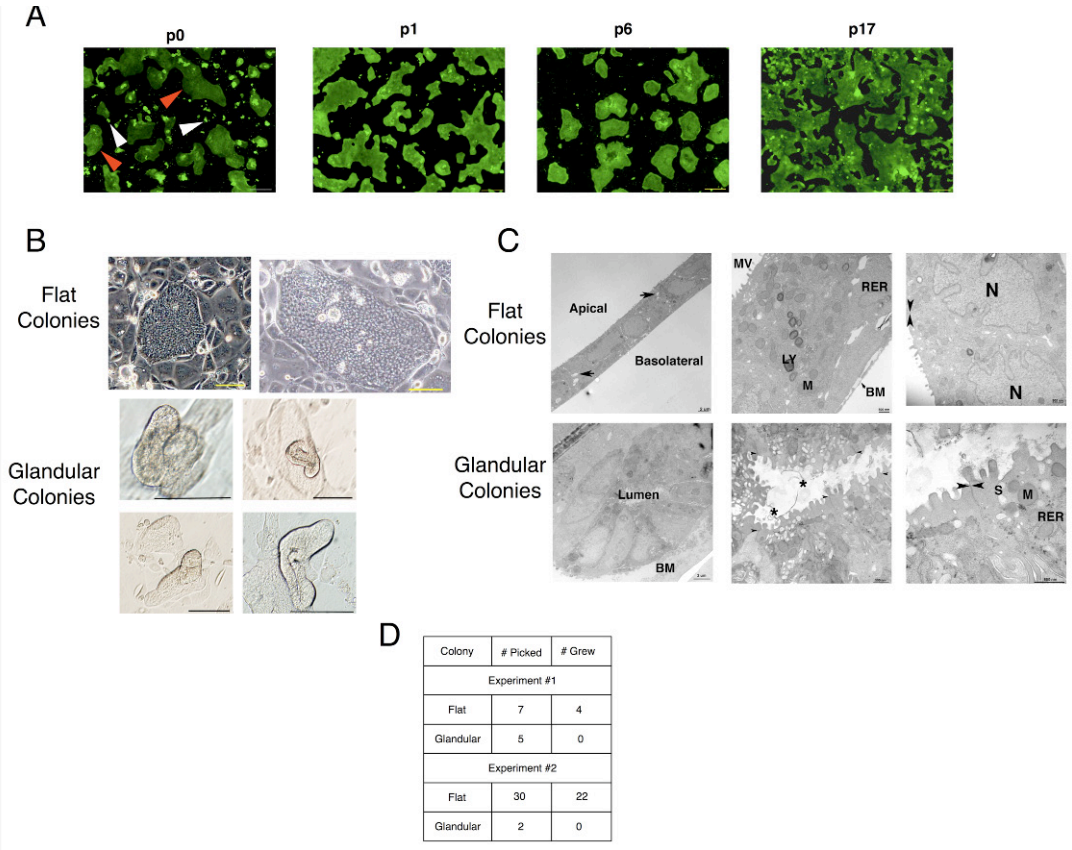
epithelial cells expand in the LA7 culture system. d0: primary cells; p0: cells at first expansion. Feeder cells (EpCAM-GFP-) have been gated out of EpCAM vs. CD45/TER119 plot for the cells at first expansion. (B) Flow cytometric analyses of expanded gallbladder cells (p0) show that all EpCAM+ cells are CD49f+. LA7 feeder cells are the EpCAM-GFP- cells in middle plot. (C) Limiting Dilution Analyses were carried out on EpCAM+CD49f^{hi} and EpCAM+CD49^{lo} cells from primary gallbladder. The Colony Forming Unit (CFU) frequency±SE (L-Calc®) indicates that CD49f enriches for stem cells. Pearson's χ^2 statistic was calculated on the 49f^{hi} and 49f^{lo} groups by ELDA. Exp#1: χ^2:17.8; p-value: 2.43e-5. Exp#2: χ^2:42.9; p-value: 5.79e-11. Crosshairs on the flow plot in figures A to C indicate where the control population was present in the lower left hand corner. (D) Index sorts from primary gallbladder. Single EpCAM+ cells were sorted into each well of 3 96-well plates. (i) Data from 3 representative wells that grew showing CD49f expression and subsequent colony morphology. (ii) Table showing index sort results. 11/12 (91.7%) of the wells that grew originated from CD49f^{hi} cells. Scale bars: 100μm.

2.3.5 Expanded Gallbladder cells Exhibit Morphological Heterogeneity

We observed the formation of two distinct types of colonies in EpCAM+CD49f+ gallbladder cultures at p0. The first type consisted of large colonies with an undifferentiated phenotype comprising small cells with a large nuclear-cytoplasmic ratio (red arrowheads, Figure 6A; Figure 6B). We termed these the "flat colonies". The second type was smaller more organized colonies called "glandular colonies" with an organotypic phenotype consisting of cells organized around a lumen (white arrowheads, Figure 6A; Figure 6B). Flat colonies were more

numerous than glandular ones. TEM on the flat colonies revealed a single layer of cuboidal epithelial cells (Figure 6C). These cells have defined apical-basolateral polarity, apical microvilli and appear to secrete basement membrane at their basolateral surface. They also have interdigitating lateral membranes and junctional apparatus typical of gallbladder epithelial cells.

Conversely, the glandular colonies consist of columnar epithelial cells organized around a central lumen (Figure 6C) and exhibit junctional apparatus. Unlike flat colonies, numerous secretory granules are seen in their apical cytoplasm and secretory products are present in their lumen (Figure 6C). The flat and glandular colonies are distinct by morphology and ultrastructure. Importantly, only the flat colonies are observed at late passages (Figure 6A**)** indicating that the glandular colonies are not capable of long-term self-renewal (>p3). To test this hypothesis, we passaged single colonies from p0 cultures. None of the glandular colonies could be successfully re-passaged (Figure 6D). This suggests that serial passage of the gallbladder cells past the first expansion enriches for EpCAM+CD49f+ cells that form flat colonies. As we found no additional markers to further purify gallbladder stem cells, we hypothesized that the cells past the first expansion are candidate stem cells. To determine their stemness, we tested whether the expanded EpCAM+CD49f+ gallbladder cells could satisfy the stem cell criteria of clonogenic self-renewal and lineage commitment.



D

Colony	# Picked	# Grew
Experiment #1		
Flat	7	4
Glandular	5	0
Experiment #2		
Flat	30	22
Glandular	2	0

Figure 6 EpCAM+CD49f+ gallbladder cells exhibit morphological heterogeneity in vitro

(A) At first passage (p0) *in vitro*, gallbladder cells exhibit two distinct morphologies. Flat colonies (red arrowheads) and glandular colonies (arrowheads) are observed. Only flat colonies are observed at later passages (p1, p6, p17). (B and C) Light and electron micrographs showing flat colonies and glandular colonies. Flat colonies consist of cuboidal cells with defined apical to basolateral polarity, show putative fluid transport (arrows) and have junctional apparatus typical of gallbladder epithelial cells. Glandular colonies consist of columnar cells organized around a central lumen. Secreted product (*) is observed in the lumen. (D) To determine if glandular colonies can expand past first passage, colonies were picked from first expansion (p0) culture. Only flat colonies expand past p0. MV: Microvilli, RER: Rough Endoplasmic Reticulum, LY: Lysosome, M: Mitochondria, S: Secretory granule, N: Nucleus, BM: Basement

membrane. Arrowheads: Tight Junctions. * Glycocalyceal substance. Unless specified otherwise, scale bars: 100μm.

2.3.6 EpCAM+CD49f+ cells differentiate into gallbladder-like structures *in vitro*

We developed a novel *in vitro* differentiation assay by utilizing the basement membrane extracellular matrix Matrigel. Matrigel has been shown to promote or maintain the differentiation or 3D morphogenesis of numerous cell lines and primary cells, including hepatocytes and IHBD cells (107, 121, 122). In our assay, expanded EpCAM+CD49f+ gallbladder cells (>p1) were mixed with serum-free media and layered above with matrigel (Figure 7A). Within one week, we noticed the formation of two distinct morphogenetic structures – ductular structures that adhered to the plastic (Figure 7B) and cysts that were suspended in the matrigel (Figure 7C). Both structures persisted for over six weeks in culture. The matrigel was removed from the plastic to confirm that the cysts were suspended in it. Similar morphogenesis was observed with primary gallbladder cells.

The ductular structures consisted of ball shaped interconnecting ducts. Confocal microscopy on ductular structures in matrigel showed that they are hollow (Figure 7B ii). The cysts similarly consisted of the hollow ball shaped structures, but lack interconnecting ducts and had much larger lumen (Figure 7C). They appeared early on in culture –around two or three days post plating – and expanded over time. TEM studies show that the cysts exhibit similar ultrastructure as primary mouse gallbladder (Figure 7D).

We then tested if the ductular structures and cysts represent two different morphogenetic programs. Ductular structures and cysts were separated and LDAs were performed where the cells were sorted back into matrigel or on the LA7 feeder cells. When sorted back into matrigel,

ductular structures could re-form ductular structures and cysts, and cysts were able to re-form both structures as well. In addition, both expanded equally well on the feeders and no differences in LDA were observed. Last, we performed the same assay in borosilicate dishes that inhibit cell attachment. We found that only cysts formed, which when passaged, could form both cysts and ductular structures. Therefore, ductular structures and cysts do not represent separate morphogenetic programs. Their appearance might be a function more of their microenvironment –attached to plastic vs. suspended in the matrigel – than intrinsic differences.

The physiological function of the gallbladder is to concentrate the bile and regulate its content by secretory processes (5, 103). These functions are in part due to multidrug resistance (MDR) proteins. Rhodamine 123, an MDR substrate, has been shown to accumulate in the lumen of cysts formed by a hepatic progenitor cell line grown in matrigel (107). We reasoned that such a transport assay would also be indicative of function for the gallbladder cells. Rhodamine 123 was added to the media of the matrigel cultures and confocal images were taken at various time points. We observed the steady accumulation of the dye in the lumen of the cysts over the course of 1 hour (Figure 7E). This transport was blocked by addition of Verapamil, an MDR inhibitor. These data indicate that the cysts transport the dye from their basal side into the lumen, thereby recapitulating a transport function of the gallbladder.

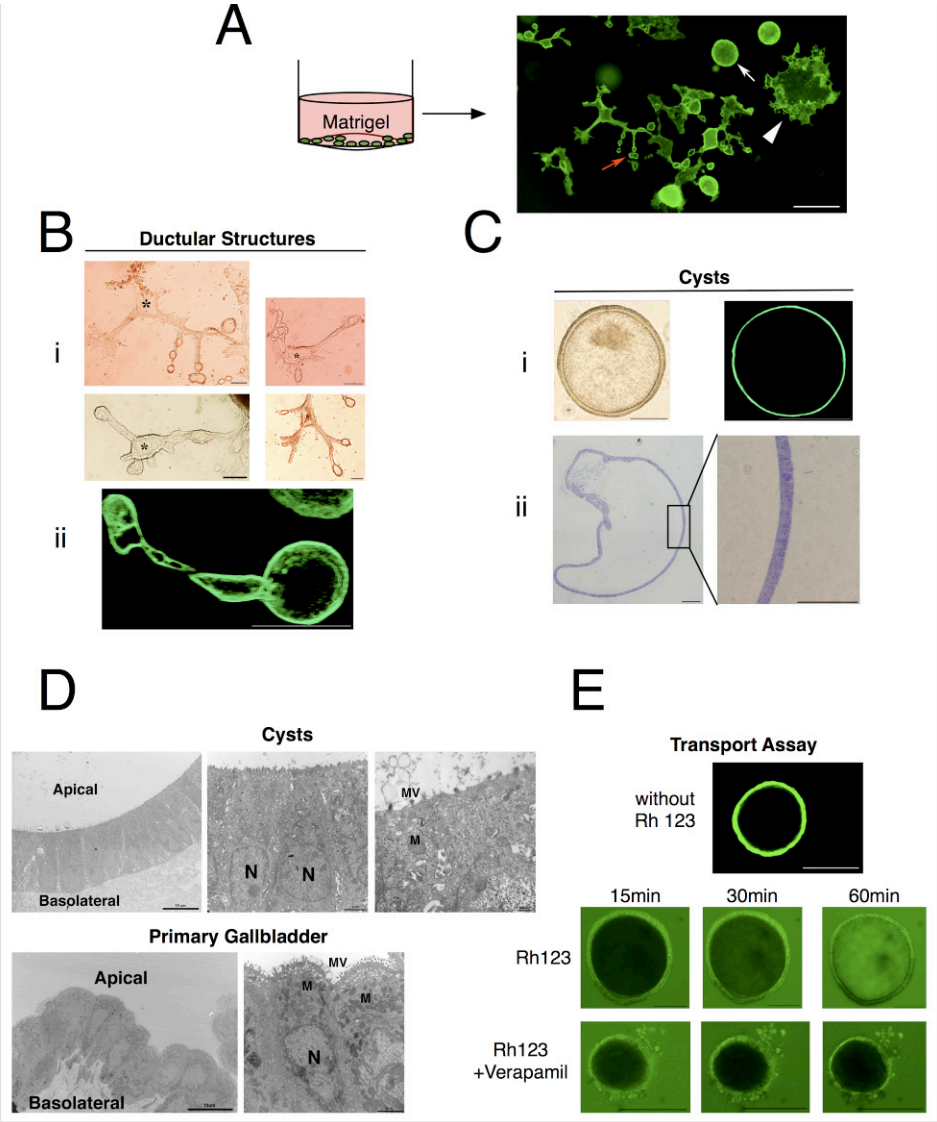


Figure 7: Expanded EpCAM+CD49f+ cells form in vitro gallbladders

(A) Schematic of *in vitro* differentiation assay. EpCAM+CD49f+ gallbladder cells (>p1) were plated on tissue culture treated dishes in serum-free media and layered on top with matrigel. Two different forms of morphogenesis were observed: ductular structures (red arrow) and cysts (white arrow). Also colonies of cells were observed growing on the plastic (white arrowhead). (B) Ductular structures are luminal, consisting of long interconnecting ducts lined by a single or double layer of cells. (i) Light micrographs showing interconnecting ducts. * Lumen. (ii) Confocal micrograph of live ductular structure *in situ* in the matrigel. (C) Cysts similarly consist

of a single or double layer of cells surrounding a hollow lumen. (i) Light and confocal micrographs of live cysts *in situ* in the matrigel, showing a hollow lumen lined by cells. (ii) Cysts were isolated from matrigel and stained with toluidine blue. (D) Electron micrographs of cysts (21 days post plating) and primary gallbladder indicating similar ultrastructure. Both consist of columnar epithelial cells with apical to basolateral polarity, basal nuclei, microvilli and junctional apparatus. (E) Cysts were incubated with 100μM Rhodamine 123 (Rh 123) ± 10μM Verapamil. Confocal micrographs showing time-lapse images of optical sections at indicated times. Rh 123 accumulates in lumen over 1 hr. Transport is blocked with Verapamil. MV: Microvilli, M: Mitochondria, N: Nucleus. Unless specified otherwise, scale bars: 100μm.

2.3.7 EpCAM+CD49f+ gallbladder cells can self-renew and differentiate from single cells

In order to test clonogenic self-renewal of EpCAM+CD49f+ gallbladder cells, we sorted single cells into 384-well plates seeded with LA7 feeders and imaged every well to confirm the presence of the single cell (Figure 8A). In this manner, 5 clonal gallbladder cultures were generated. Further analyses were carried out with Clone B21 and Clone N12 (Figure 8A). Surface marker analyses indicated that both Clone B21 and N12 have the same phenotype as the parent gallbladder cell population (Table 2) suggesting that the clones are able to recapitulate the heterogeneity of the parent. In addition, both Clone B21 and N12 exhibit the same morphogenesis in matrigel as the parent (Figure 8B).

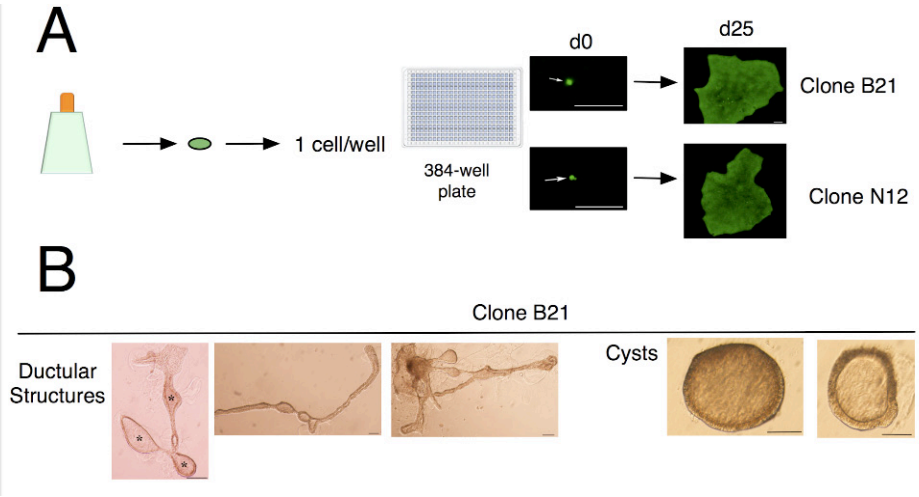


Figure 8: Expanded EpCAM+CD49f+ cells undergo clonogenic self-renewal

A) Schematic of single-cell clonogenic assay. EpCAM+CD49f+ gallbladder cells at first passage (p0) were sorted into 384-well plates at one cell/well. In this manner, 8 clones were generated. Representative images of CloneB21 and CloneN12 are depicted. (B) Clonal gallbladder cultures were expanded further and differentiation assays were carried out. Clones exhibit similar morphogenesis compared to parent cultures. * Lumen, Scale bars: 100μm.

2.3.8 Expanded EpCAM+CD49f+ cells can engraft *in vivo*

We then determined if the expanded EpCAM+CD49f+ cells could survive and engraft *in vivo*. An ideal location for engraftment would be the native gallbladder. However, as there currently are no protocols that allow for the injection and maintenance of cells in the gallbladder, we attempted engraftment at an ectopic location. Okumura et al. (123) have reported the long-term engraftment of *in vitro* explants of human gallbladder in the subcutaneous space of athymic nude mice. We injected the expanded EpCAM+CD49f+ cells mixed with matrigel into the subcutaneous neck region of immunodeficient mice. We observed the formation of cyst-like structures in the subcutaneous space one week post-injection (Figure 9A). These cysts consisted

of cells organized around a central lumen. Seven out of seven (100%) mice injected formed cysts. However, engraftment was short term. Only one out of three (33%) mice exhibited cyst formation at two weeks. Similar results were obtained with clonal cultures.

We then isolated the cells from the cysts *in vivo* two weeks post injection and cultured them *in vitro* to test their ability to reinitiate cultures with stem cell properties. Flow cytometry analyses showed that the cells isolated from cysts *in vivo* were EpCAM+CD49f+. These cells re-expanded *in vitro* on the feeder cells forming colonies that were morphologically identical to the parent and clonal cultures and remained EpCAM+CD49f+ (Figure 9B). These data indicate that the expanded EpCAM+CD49f+ cells survive and engraft *in vivo*, while retaining their proliferative ability *in vitro*.

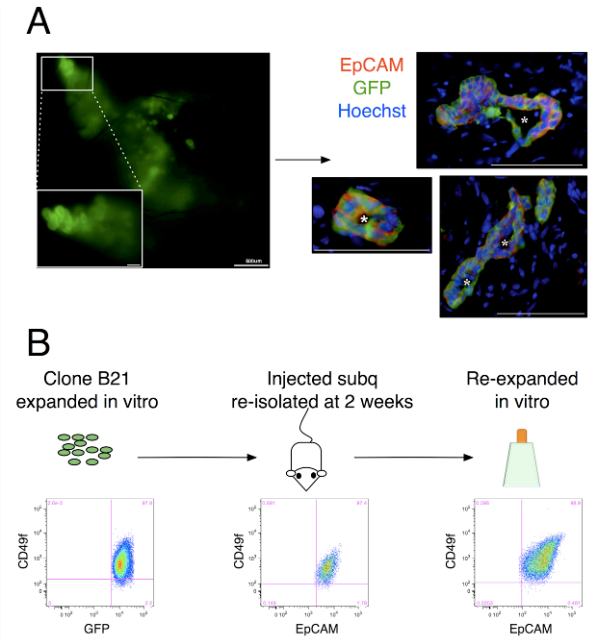


Figure 9: Expanded EpCAM+CD49f+ cells engraft in vivo

Parent or clonal gallbladder cultures were mixed with matrigel and injected into the subcutaneous (subq) space of Rag2$^{-/-}\gamma C^{-/-}$ mice. Cysts were observed at 1 week and 2 weeks post injection. (A) (Left Panel) Macroscopic view of matrigel isolated at 1week from mouse

injected with parent EpCAM+CD49f+ cells (right panel) Sections of same matrigel stained with EpCAM and GFP, showing that cysts are hollow and EpCAM+. Cysts were seen in at one week in 7/7 and 2/3 mice injected with parent and clonal cultures respectively. 1/5 and 1/3 mice respectively, exhibited engraftment at 2 weeks. (B) Clonal gallbladder cells remain EpCAM+CD49f+ *in vivo*. Expanded gallbladder cells from CloneB21 were injected subq. At 2 weeks, the matrigel was removed and cells re-isolated. Flow cytometric analysis shows that re-isolated cells are EpCAM+CD49f+. These cells were expanded *in vitro* on feeder cells. Flow cytometric analysis at p1 (second passage) showing that they remain EpCAM+CD49f+. Crosshairs on the flow plot in figures A to C indicate where the control population was present in the lower left hand corner. Debris and cell aggregates were gated out in each plot. In the middle plot, GFP- cells were gated out as well. *: Lumen. Unless specified otherwise, scale bars: 100μm.

2.3.9 Gallbladder cells are unique compared to IHBD cells

There is evidence indicating that intra- and extrahepatic bile duct cells develop separately (20). To date there are no reports of the molecular differences –if any- between IHBD and gallbladder cells. We first screened primary IHBD cells with the same antibody panel used for the primary gallbladder cells (Table 2). Most IHBD cells express EpCAM (114), and we used EpCAM expression to separate IHBD cells from other liver cells. Briefly, following liver perfusion of GFP+ mice, the high spin fraction was separated and used to isolate IHBD cells.

Interestingly, we found differences in integrin expression, including CD49f (Figure 10A). Other notable markers that showed differences between IHBD and gallbladder cells were CD49e, CD81, CD54, CD26 and CD166 (Figure 10A). We then determined if expanded

gallbladder cells and IHBD cells were different. The IHBD cells are capable of expansion on the LA7 feeders and the feeder cells select for EpCAM+ cells (Figure 10B). In addition, IHBD cells form flat colonies similar to the gallbladder cells. The phenotypic profiles of the IHBD and gallbladder cells converged in culture and we did not detect any differences using the foregoing panel of antibodies. Consequently, we used oligonucleotide microarrays to test for expression differences between the two cell types.

We have shown that the expanded gallbladder cells or EpCAM+CD49f+ cells are capable of self-renewal and lineage commitment. It is possible that the expanded IHBD cells might satisfy these requirements as well. However, the evaluation of IHBD stem cells belongs to a different study and we focused on the differences in the transcriptomes of the expanded gallbladder and IHBD cells. Briefly, expanded gallbladder cells and IHBD cells were separated from LA7 feeder cells using magnetic activated cell sorting (Table 1). Differential gene expression between expanded gallbladder and IHBD cells (fold change ≥2) were calculated by Significance Analysis of Microarrays (SAMs) (111) using a false discovery rate of 10%. In this manner, we found 64 genes to be upregulated in IHBD cells (Figure 10C) including those involving lipid metabolism (8 genes), stem cell proliferation (3 genes) and drug metabolism (2 genes) (Table 3). Notable genes or groups of genes that were different were cytochrome P450, Indian hedgehog, glutathione-S-transferase and solute carrier families 22, 26, 37 and 45. These differences indicate that the expanded gallbladder and IHBD cells have distinct transcriptomes and suggest functional differences as well.

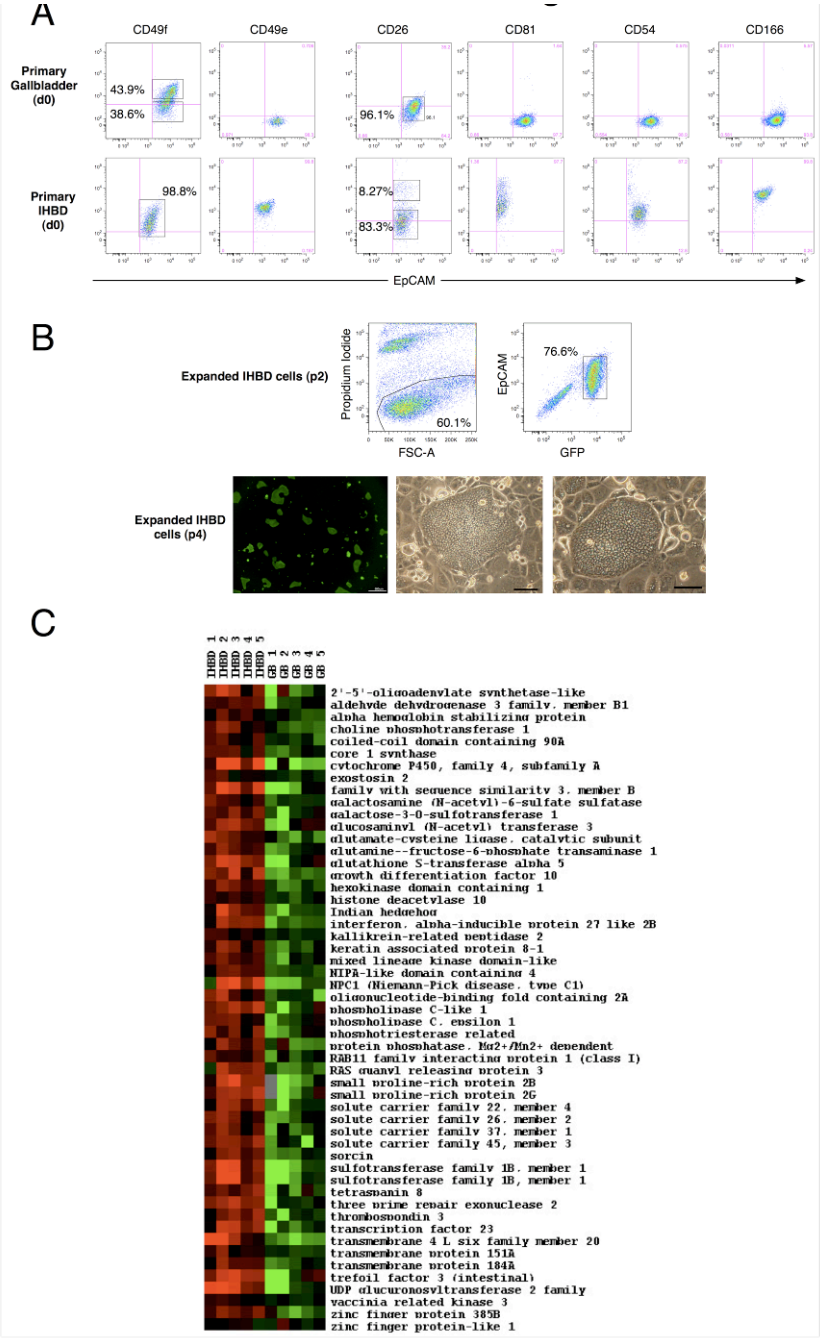


Figure 10: Expanded EpCAM+CD49f+ cells are unique compared to IHBD cells

(A) Flow cytometric analyses of EpCAM+ (epithelial) cells from primary gallbladder and primary liver. d0: primary cells. Phenotypic differences with select cell surface markers suggest that gallbladder and IHBD cells are different. Crosshairs on the flow plot in figures A to C indicate where the control population was present in the lower left hand corner. (B) IHBD cells

expand on LA7 feeders and exhibit similar morphology to expanded gallbladder cells. (Upper panel) Flow cytometric analyses of expanded IHBD cells (p2) indicate that all GFP+ cells are EpCAM+. (Lower panel) Expanded IHBD cells exhibit flat colony appearance. (C) Heatmap of 53 genes with known annotations and fold changes (≥2) differentially expressed between IHBD and gallbladder cells. Data were imported in Cluster and heat maps were generated with TreeView (http://rana.lbl.gov/EisenSoftware.htm). Black represents genes whose expression was at the mean intensity; red represents intensities that are greater than the mean; green represents intensities that are less than the mean. Unless specified otherwise, scale bars: 100μm.

Table 3: Predictive functional analysis of 2-fold differentially expressed genes

The 64 genes with fold changes greater than or equal to 2 were analyzed in Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). The functional analysis identified the biological functions and/or diseases that were most significant to the dataset. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that dataset is due to chance alone. In this analysis only $p<0.0025$ were considered.

Category	Molecules	# Molecules	Functions Annotation	p-Value
Lipid Metabolism	ALDH3B1,CHPT1,CYP4A22,GAL3ST 1,NPC1L1,PLCE1,SLC22A4,UGT2B17	8	metabolism of lipid	2.63E-04
Small Molecule Biochemistry	ALDH3B1,CHPT1,CYP4A22,GAL3ST 1,NPC1L1,PLCE1,SLC22A4,UGT2B17	8	metabolism of lipid	2.63E-04
Cardiovascular System Development and Function	GCLC,IHH	2	size of blood vessel	4.69E-04
Organismal Development	GCLC,IHH	2	size of blood vessel	4.69E-04
Tissue Morphology	GCLC,IHH	2	size of blood vessel	4.69E-04
Post-Translational Modification	GAL3ST1,GCNT3,TSPAN8	3	glycosylation of protein	8.02E-04
Cellular Growth and Proliferation	GALNS,IHH,TREX2	3	proliferation of stem cells	1.29E-03
Drug Metabolism	GCLC,GSTA5	2	metabolism of glutathione	2.38E-03

2.4 DISCUSSION

Little is known about the resident stem cells in gallbladder and the relationship between the stem cells of the hepato-biliary system. Our goal here was to identify and characterize stem cells in the adult mouse gallbladder. We found that an EpCAM+CD49f^{hi} subpopulation from primary mouse gallbladder can expand from single cells, and exhibits morphogenesis in organotypic culture *in vitro*. Both parent and clonal cultures were capable of survival and short-term morphogenesis in an adapted *in vivo* assay. We therefore concluded that EpCAM+CD49f^{hi} gallbladder cells satisfy the stem cell criteria of clonogenic self-renewal and lineage commitment and represent a gallbladder stem cell population. Last we determined that gallbladder stem cells and IHBD cells expanded *in vitro* have distinct transcriptomes, suggesting that the cells of the IHBD and EHBD systems are different.

This study is the first to describe the identification and prospective isolation of stem cells from an uninjured mouse gallbladder. Previous reports of stem cells in the EHBD system have focused on injury models (38) or disease conditions such as biliary atresia (124, 125). Furthermore, these studies do not distinguish epithelial from non-epithelial cells in their isolation protocols. We used EpCAM to isolate gallbladder epithelial cells thereby preventing contamination by non-epithelial cells. This is especially important as we detect EpCAM-CD49f+ cells in primary gallbladder by both immunohistochemistry and flow cytometry. The isolation and subsequent expansion of EpCAM+CD49f+ cells thereby allows for the definitive identification of resident epithelial stem cells.

We confirmed CD49f as a gallbladder stem cell marker by LDAs and index sorts from primary gallbladder. EpCAM+CD49f^{hi} cells have a significantly higher CFU readout relative to EpCAM+CD49f^{lo} cells. The low enrichment in CFU readout indicates that additional markers

are required to further purify stem cells such that single cells can be isolated and expanded (126). Therefore, expression of EpCAM and CD49f enriches, but does not select for stem cells. All gallbladder epithelial cells expanded *in vitro* were EpCAM+CD49f+. However, these cells exhibited morphological heterogeneity at first expansion, forming flat and glandular colonies. Interestingly, none of the glandular colonies and only a fraction of the flat colonies were capable of serial passage. It appears that the EpCAM+CD49f^{hi} population in primary gallbladder is itself heterogeneous with only a subpopulation of cells capable of self-renewal. We could not identify any additional markers to select for this specific subpopulation directly from primary tissue and therefore characterized the stemness of the EpCAM+CD49f+ cells expanded past p0.

We determined that the expanded EpCAM+CD49f+ cells can self-renew clonogenically. However, defined protocols for gallbladder epithelial cell differentiation do not exist. In the past, researchers have used collagen gel sandwich culture to observe cyst morphogenesis with rabbit gallbladder epithelial cells (127, 128). The collagen gel is supplemented with exogenous growth factors such as EGF and TGFβ1. We postulate that our 3D culture system is similar to collagen gel culture in that matrigel is an appropriate growth factor containing extracellular matrix that supports morphogenesis. Cyst formation in our culture is similar in morphology and ultrastructure to that observed before (127, 128). We also observed dye transport that is reminiscent of a transport function of the gallbladder. In addition, we observed similar morphogenesis *in vivo* after transplantation. We chose an ectopic location, as engraftment in the native gallbladder would be technically challenging and the subcutaneous space has been shown to engraft human gallbladder cells (123). Lee et al. (83) have shown that gallbladder cells can engraft into the native liver of SCID mice. However, engraftment was significant only with

tremendous injury to the liver (retrorsine and partial hepatectomy or carbon tetrachloride treatment) and required very large numbers of cells. For these reasons, we concluded that subcutaneous rather than liver engraftment would be a more apt *in vivo* assay. In our hands, the EpCAM+CD49f+ cells only engraft in the short term (2 weeks post transplantation). This short-term engraftment might be due to a lack of growth stimulus in the recipient. Also under physiological conditions, the rate of cell proliferation in the gallbladder epithelium is low (37). Future studies will determine if long-term engraftment of EpCAM+CD49f+ cells is possible. For now, these data conclusively show that parent and clonal EpCAM+CD49f+ cells can organize into organotypic structures that mimic the morphology, ultrastructure and function of the native gallbladder, both *in vitro* and *in vivo*.

Spence et al. (20) have recently showed that IHBD and EHBD cells develop from separate precursors. However, there are no reports describing their similarities or differences in the adult. We found that expression of CD49f, CD49e, CD81, CD26, CD54 and CD166 was different between primary IHBD and gallbladder cells. The goal of our experiment was to evaluate the differences if any between gallbladder stem cells and IHBD cells. Expanded EpCAM+CD49f+ gallbladder cells (>p0) represent a purer stem cell population than primary EpCAM+CD49f^{hi} cells. The latter forms both flat and glandular colonies and only a fraction of the flat can self-renew. Therefore, we ran microarray analyses on expanded EpCAM+CD49f+ cells (>p1) and expanded IHBD cells. The major groups of differentially expressed genes were cytochrome P450 genes, glutathione-S-transferase and the solute carrier family genes. Also interferon (IFN)-inducible protein 27 was differentially expressed between gallbladder and IHBD cells. Interestingly, expression of CD54 is known to be immunologically mediated (129). The immunologic properties of bile duct cells have long been considered. They are the primary

site of damage in inflammatory diseases such as primary biliary cirrhosis (130) and biliary atresia (21) and in liver allograft rejection (131). The differential expression of an IFN-inducible protein and CD54 indicates that the immunologic properties of IHBD and gallbladder cells could be different. Studies of IHBD cells are hindered by a technical inability to isolate and expand them from primary tissue (8, 103). We circumvented this hurdle by using LA7 feeder cells that allow for a robust expansion of IHBD epithelial (EpCAM+) cells. This expansion assay along with the 3D matrigel assay could serve as interchangeable and technically easy tools to study bile duct cells. In all, the complete elucidation of the differences between the IHBD and gallbladder cells belongs to another study, as does the evaluation of the stem cell characteristics of the expanded IHBD cells. The focus of this manuscript was to isolate and characterize gallbladder stem cells.

We postulate that this study will have important clinical significance. Gallbladder stem cells could be used to treat biliary atresia as has been noted with hepatic progenitor cells (132). These cells could also be reprogrammed into hepatocytes or endocrine cells. There have been recent reports of the differentiation of gallbladder epithelial cells into hepatocytes (81, 83) and ectopic endocrine cells have been observed in the EHBD cells of *Hes1*$^{-/-}$ deficient mice (13). Gallbladder stem cells might be capable of such plasticity, which along with the ready availability of donor tissue would make them an attractive candidate for cell-based therapy.

3.0 ISOLATION AND EXPANSION OF A UNIPOTENT IHBD STEM CELL POPULATION FROM ADULT MOUSE LIVER

3.1 INTRODUCTION

The identification of liver stem cells holds great promise for both basic biology and cell-based therapy to treat end stage liver disease. The liver consists of two parenchymal and epithelial cell types: hepatocyte and IHBD cells, and similar to other adult stem cell systems, a prerequisite of a liver stem cell is to generate both cell types, i.e. be bipotent. However, the origin of hepatocytes and IHBD cells during normal homeostasis is still debated (57, 59, 60, 86). Hepatocytes themselves have a remarkable ability to proliferate following injury such as partial hepatectomy (133). It is believed that stem cells do not have a significant role to play in tissue repair and maintenance unless this foregoing ability of hepatocytes is blocked (58). The identification of resident stem cells in the uninjured liver has accordingly been controversial.

There have been numerous reports of the prospective identification of bipotent stem cells in the uninjured mouse and human livers (63-73). Along these lines, there have been many candidate cell surface markers identified that enrich for liver stem cells. However, many of these markers also co-stain with IHBD cells. Therefore, it is not clear if the liver stem cells identified represent a separate population from IHBD cells and hepatocytes or a subpopulation of IHBD cells themselves. In addition, *in vitro* expanded stem cells are not homogeneous but

contain contaminating mesenchymal and hematopoietic cells, which makes discernment of a resident epithelial stem cell difficult. Finally, the hepatocyte differentiation observed with these cells was inconclusive and occurred at very low frequencies. This could be for various reasons, beginning with the lack of a definitive *in vitro* hepatocyte differentiation protocol. However, it could also be because these candidate stem cells are not capable of *de novo* hepatocyte differentiation.

Stem cells are primitive cells that exhibit single cell self-renewal and lineage commitment (91, 134). We have previously developed *in vitro* assays for the evaluation of resident stem cells in the adult mouse gallbladder (135). In this study, we adapt these assays to evaluate single cell self-renewal and lineage commitment of IHBD cells. There are no cell surface markers for the prospective isolation of candidate liver stem cells in the uninjured liver. However, IHBD cells can be separated from hepatocytes, mesenchymal and hematopoietic cells in primary liver. Therefore, we chose to isolate mouse IHBD cells and characterize them using the assays previously developed for gallbladder stem cells. Last, we evaluated bipotentcy of candidate IHBD stem cells assessing *de novo* hepatic differentiation after by transplantation into the *Fah*$^{-/-}$ mouse.

3.2 MATERIAL AND METHODS

3.2.1 Isolation of IHBD cells

IHBD cells were harvested using the 2-step collagenase perfusion technique introduced by Seglen et al. (109). Following isolation, hepatocytes were separated by a low speed centrifugation (50g, 2min). The resulting supernatant was subjected to two more low speed centrifugations followed by a high speed centrifugation (300g, 5min). The resulting cell pellet was termed the small cell fraction.

3.2.2 MACS enrichment of EpCAM+ IHBD cells

Small cell fractions were stained with either EpCAM-PE or EpCAM-Biotin (BioLegend; Dilution 1:50) primary antibodies, followed by the appropriate microbead conjugated secondary antibody (Miltenyi Biotec) and eluted through a MS MACS® separation column (Miltenyi Biotec). The number of microbead conjugated secondary was adjusted for the low cell frequency of EpCAM+ cells. We did this by recalculating the total cell number. First we assumed that at least 50% of the actual number of total cells were EpCAM+ cells and this number was 5% of the recalculated total cells. We then adjusted the volume of microbeads according to this recalculated total cell number. Resulting MACS enriched (MACS+) fraction were grown on lethally irradiated feeder cells as described previously (135).

3.2.3 Immunofluorescence

Antibodies against EpCAM, CK19 and Albumin (Bethyl Laboratories, Dilution 1:50) were used at the appropriate dilution in PBS (**Table 1**). Sections were blocked with 0.5% milk and stained with either anti-rat or anti-rabbit Alexa Fluor® secondaries (Invitrogen, CA). Images were taken using an IX71 Inverted microscope (Olympus, PA).

3.2.4 Intracellular staining with CK19

Small cell fractions were stained with the appropriate primary antibodies (**Table 1**) and fixed in 4% PFA for 10mins. These fractions were washed in PBS and permeabilized in either 0.3% Saponin or 0.1% Triton-X for in 0.5% BSA for 10mins, and stained with Rabbit anti-mouse CK19 antibody (kind gift of Dr. Markus Grompe, Portland OR) in the respectively permeabilization solution for at least 1 hour. Following this, the fractions were stained with the respective secondary antibodies, and analyzed on the BD FACSCanto.

3.2.5 Injection into FAH mice

Cell suspensions were prepared in 50µl of HBSS and injected intrasplenically into *Fah*$^{-/-}$ mice. Animals were anesthetized and a small surgical incision was made in the left flank. The spleen was exposed and the injection was made into the inferior pole of the spleen using a 28-gauge needle. The injection site was ligated to prevent cell leakage and bleeding. All mice were kept on NTBC until transplantation. NTBC was removed just after transplantation. The weight of the mice was monitored weekly to determine their health. *Fah*$^{-/-}$ mice lose weight during the first

few weeks after transplanation due to gradual loss of liver function and progressively regain their initial weight later when donor liver cells regenerate liver tissue and hepatic functions. Whenever the animals lost more than 25% of their initial body weight, NTBC was given back to restore liver function. It usually took five to seven days for the mice to return to the initial weight and liver functions under NTBC. At that point, NTBC is removed again to induce liver failure. Such a protocol has previously been used to allow a low number of cells to engraft in the liver cells (72, 136).

3.3 RESULTS

3.3.1 Isolation of EpCAM+ IHBD cells

In order to characterize gallbladder stem cells, we have previously used EpCAM to identify gallbladder epithelial cells (135). EpCAM is an epithelial cell surface marker expressed on keratinocytes and thymic epithelial cells (137). There are various reports indicating that EpCAM is expressed on IHBD cells (114, 138). To investigate these data, livers were perfused by the standard two-step collagenase protocol introduced by Seglen et al. (109). Briefly, livers were perfused with collagenase and following cell isolation, hepatocytes and small cells were separated by centrifugation (Figure 11). In our experiments, hepatocytes constituted ~65% of the total cells isolated. The small cell fraction was subsequently labeled with EpCAM, hematopoietic (CD45), endothelial (CD31) and macrophage (F4/80) cell markers for FACS. Analysis of these cells indicated three distinct populations of cells: CD45+EpCAM- (hematopoietic cells), CD45-EpCAM- (non-hematopoietic and non-epithelial cells) and CD45-

EpCAM+ (epithelial cells). CD45-EpCAM+ cells were CD31-F4/80- (Figure 12) and represented the smallest fraction within the small cells (~1%). Cytospins of the CD45-EpCAM+ cells indicate that almost all are CK19+ (Figure 11B). Since CK19 is considered to be an IHBD cell marker (114), we concluded that EpCAM would be a good cell surface marker to identify IHBD cells. Analysis of the CD45-EpCAM+ cells indicated that they express the epithelial markers CD49f, CD49e, CD29, and CD133 (135) (Figure 13), confirming them as bile duct epithelial cells.

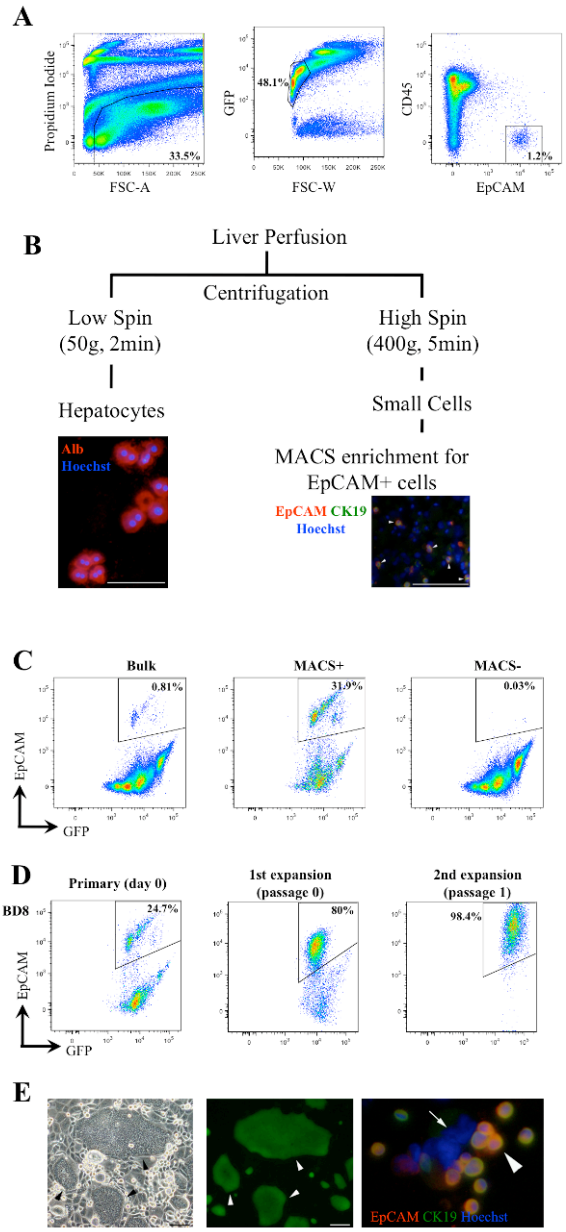


Figure 11: Isolation and expansion of EpCAM+ IHBD cells

(A) Flow cytometric analysis of the small cell fraction from liver perfusion indicating a small (≤1%) EpCAM+CD45- cell fraction representing IHBD cells. (B) Strategy for enrichment of EpCAM+ IHBD cells from small cell fraction. MACS+ fractions were stained with CK19 to confirm enrichment of IHBD cells. Note that not all cells in MACS+ fractions are

EpCAM+CK19+ indicating the presence of possible hematopoietic and mesenchymal cells. The large cell fraction was stained with Albumin to confirm hepatocytes. (C) Representative analysis of a MACS experiment on a single IHBD sample. The data clearly indicate a strong enrichment of EpCAM+ cells. (D) MACS+ fractions were expanded on rat feeder cells. Representative analysis of a single expanded IHBD sample indicating clear selection of EpCAM+ cells from primary to first expansion (p0) and second expansion (p1). (E) Brightfield and epifluorescent micrographs of EpCAM+ IHBD cells *in vitro* (passage 2) (black arrowheads point to colonies). Cytospins of expanded EpCAM+ IHBD cells stained with EpCAM and CK19. White arrowheads indicate EpCAM+CK19+ IHBD cells. White arrows indicate rat feeder cells. Scale bars: 100 μm.

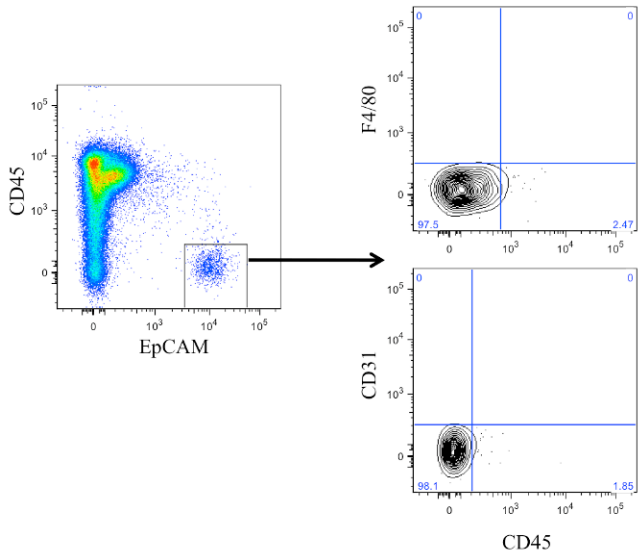


Figure 12: Analysis of hematopoietic and mesenchymal markers on primary EpCAM+ IHBD cells

Primary EpCAM+ IHBD cells were analyzed by flow cytometry for hematopoietic markers, CD45 and F4/80 and the endothelial marker, CD31. EpCAM+ cells are CD45-, CD31- and F4/80-.

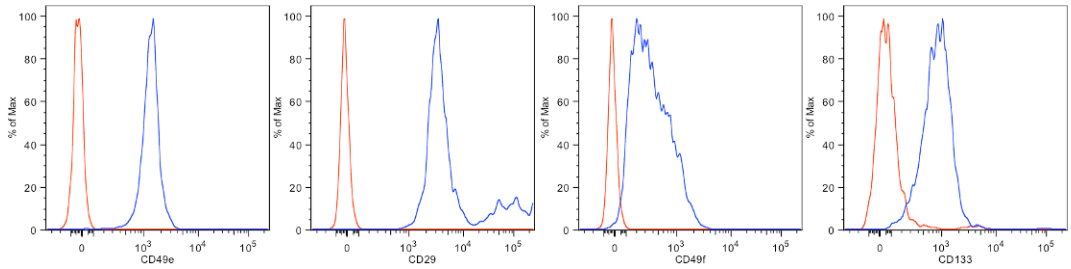


Figure 13: Epithelial marker expression on primary EpCAM+ IHBD cells

Red peaks correspond to the negative cell population, i.e. without primary antibody. Blue peaks correspond to the appropriate stained population. Primary EpCAM+ IHBD cells are CD49f+, CD49e+, CD133+ and CD29+. We were not able to detect any heterogeneity with these markers.

3.3.2 Expansion of the EpCAM+ IHBD cells

We have previously used the rat mammary tumor cell line, LA7, as feeder cells for the expansion of mouse gallbladder stem cells (135) and human colon cancer stem cells (98). These feeder cells are known to select for epithelial cell growth (95) and in our previous study, they selected for gallbladder epithelial cells (135). We therefore tested if the LA7 feeder cells would be good candidate for IHBD cell growth. Initially, when the total small cell fraction was plated on the feeder cells, we did not observe a robust and reproducible epithelial cell expansion (data not shown), possibly because of the low frequency of IHBD cells in the small cell fraction (≤1% EpCAM+ CD45- cells). We then attempted to enrich for EpCAM+ cells using Magnetic Activated Cell Sorting (MACS) prior to plating on feeder cells (Figure 11B).

Small cell fractions were stained with EpCAM followed by the appropriate microbead-conjugated secondary (see *Materials and methods*), fractionated using a column and analyzed by flow cytometry. We were able to achieve a robust enrichment of the EpCAM+ cells using

MACS (Figure 11C). Analyses of isolates from multiple liver perfusions indicated that enrichment varied from a 5-45 fold increase of EpCAM+ cells (Table 4). We obtained better enrichment from younger mice (6-9 weeks old) compared to older mice (16 weeks old). In order to expand them *in vitro*, MACS enriched cultures were plated on feeder cells and trypsinized after 4-7 weeks. Mouse cells (gfp+ cells) were separated from rat feeder cells (gfp- cells) as described previously (135). Flow cytometric analyses showed that there was no fusion between the IHBD and feeder cells (Figure 14). We observed a further two-five fold enrichment of EpCAM+ IHBD cells after the first expansion *in vitro* (Figure 11D and Table 5). These data confirm that the feeder cells select for IHBD epithelial cells. After the second expansion, almost all (>98%) of the gfp+ cells *in vitro* were EpCAM+, indicating a pure expansion of EpCAM+ IHBD cells. After immunostaining with CK19, we found that that all the expanded IHBD cells were EpCAM+CK19+ (Figure 11E). In this manner, using an anti-EpCAM enrichment protocol followed by serial passage on rat feeder cells, we achieved a robust and reproducible expansion of IHBD epithelial cells.

Table 4: MACS enrichment of Primary EpCAM+ IHBD cells

Small cell from liver perfusion were stained with EpCAM-PE antibodies followed by anti PE-conjugated microbeads and run over a single MACS column. MACS+ and MACS- negative were analyzed by flow cytometry. In all sample analyzed exhibited enrichment of EpCAM+ cells, with enrichment varying between 5-50 fold. Enrichment and yield varied depending on age of mice, with younger mice giving a smaller but purer EpCAM+ cell population.

Population	**% Primary EpCAM+ gfp+ cells[#]**	**Fold Increase in EpCAM+ cells; Yield**	**Age/Sex**
BD1	Bulk (0.79%) MACS+ (4.5%) MACS- (0%)	5.7 fold enrichment Bulk: 4.5e6 cells MACS+: 390,000 cells	16 weeks, F
BD5	Bulk (0.65%) MACS+ (29.9%) MACS- (0.01%)	46 fold enrichment Bulk: 10.1e6 cells MACS+: 195,000 cells	7 weeks, F
BD8	Bulk (0.5%) MACS+ (24.7%) MACS-: (0.02%)	49 fold enrichment Bulk: 9e6 cells MACS+: 100,000 cells	6.5 weeks, M
BD12	Bulk (1%) MACS+ (14.9%) MACS- (0.02%)	15 fold enrichment Bulk: 12.3e6 cells MACS+: 530,000 cells	16 weeks, F
BD11	Bulk (1.1%)	30 fold enrichment	9 weeks, F

	MACS+ (31.7%)	Bulk: 12.4e6 cells	
	MACS- (0.11%)	MACS+: 109,000cells	

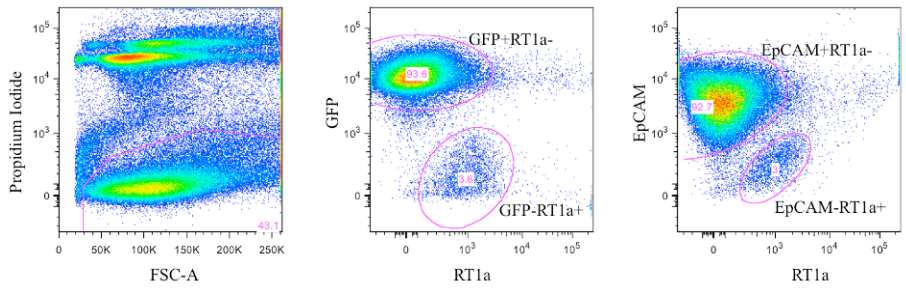


Figure 14: IHBD cells do not undergo fusion in vitro

Flow cytometric analyses of IHBD cells. RT1a is the Class I MHC antigen for the rat feeder cells. The middle and right plots are different representations of the live cells in the left plot. The data clearly indicate two distinct populations: GFP+RT1a- and GFP-RTa1+ or EpCAM+RT1a- and EpCAM-RT1a+, confirming the adequate separation of IHBD cells from rat feeder cells.

Table 5: Enrichment of EpCAM+ IHBD cells in vitro

MACS enriched fractions from primary tissue were expanded in vitro on rat feeder cells and analyzed at first expansion (p0). In all samples analyzed, we noticed a strong enrichment of EpCAM+ IHBD cells. On average, after five weeks in culture, there was a 2-5 fold enrichment of EpCAM+ cells.

Population	**% Expanded EpCAM+ gfp+ cells[#]**	**Fold Increase in EpCAM+ cells from Primary**	**Days in Culture**
BD1	21%	4.6	28
BD5	89%	3	33
BD8	80%	3.3	50
BD11	55%	1.7	42

3.3.3 Evidence of heterogeneity in EpCAM+ IHBD cells

Stem and progenitor cells are defined by their ability for self-renewal and we reasoned that *in vitro* expansion of IHBD cells would select for primitive or stem cells. To correlate IHBD cell expansion with a surface marker phenotypic profile, we screened primary and expanded EpCAM+ IHBD cells with a panel of 38 markers, similar to our previous characterization of mouse gallbladder stem cells (135) (Table 6). We observed that the expanded EpCAM+ IHBD cells remained positive for CD49f, CD49e, CD29, and CD133 and were negative for lineage markers, CD45, CD31 and F4/80. However, unlike the gallbladder cells, we were not able to identify markers that highlighted functionally distinct populations of IHBD cells (Table 6). Function in this case is defined by a colony forming assay (see below). Therefore, we evaluated the colony forming ability of total EpCAM+ cells by LDAs in order to determine if all or only a subpopulation of EpCAM+ cells were capable of self-renewal.

Table 6: Phenotypic profile of primary and expanded IHBD cells

Primary and expanded EpCAM+ IHBD cells were screened with a panel of 38 cell surface markers. Of these markers, we only observed consistent heterogeneous expression of CD26 (DPPIV) and Stem cell antigen (Sca1). LDA analyses analyzing colony forming ability indicated that these markers do not highlight functionally distinct populations.

Marker	Primary IHBD	Expanded IHBD
CD2	-	-
CD3e	-	-
CD4	-	-
CD5	-	-
CD8a	-	-
CD9	+	+
CD11b	-	-
CD13	-	-
CD14	-	-
CD19	-	-
CD26	+/Het	+/Low
CD29	+	+
CD31	-	-
CD44	-	-
CD45	-	-
CD49e	+	-
CD49f	+	+
CD54	+	-
CD56	-	-
CD73	+	-
CD81	+	-
CD86		-
CD95	-	-
CD117	-	-
CD121a		-
CD132		-
CD133	+	+
CD166	+	+
CXCR4	-	-
DBA	+/Het	+/Het
Dlk	-	-
F4/80	-	-
EpCAM	+	+
Gr1.1	-	-
Sca1	Het	+
Siglec F	-	-

TER119	-	-
Qa-1	-	+/Low

LDAs quantify the frequency of the colony forming cells in a given cell population (99) and were key to the identification of hematopoietic (100) and neural stem cells (101). We have previously used LDAs to identify colony forming cells in adult mouse gallbladder (135). In this study, we sorted primary and expanded EpCAM+ IHBD cells directly onto the rat feeder cells. We observed a similar colony forming unit (CFU) frequency between primary (1 out of 22 – 1 out of 30) and expanded (1 out of 18 – 1 out of 20) EpCAM+ IHBD cells (Table 7). Chi-square tests revealed no statistical difference in the CFU frequency ± SE range between primary and expanded IHBD cells ($p>0.05$). These data clearly demonstrate the presence and long-term (>passage 3) expansion of a select sub-population of EpCAM+ IHBD cells capable of colony formation *in vitro*. However, as detailed earlier, we were not able to identify cell surface markers that would select for this sub-population. Subsequently, we chose to evaluate the stem cell properties of single cell self-renewal and lineage commitment of the expanded EpCAM+ IHBD cells.

Table 7: Evidence of heterogeneity in EpCAM+ IHBD cells

LDAs were carried out on two primary and expanded EpCAM+ cell populations respectively. CFU frequency ± SE (L-Calc®) results indicate stable *in vitro* expansion of EpCAM+ cells through late passage (> passage 3). Pearson's chi-square statistic was calculated on all the four cell populations by the online tool, Extreme Limiting Dilution Analyses (ELDA), by either keeping all cell populations separate (Case A), or by grouping the primary and expanded cell populations together (Case B). Case A: Chi-square statistic: 4.12; p-value: 0.249. Case B: Chi-square statistic: 2.47; p-value: 0.116.

	Population	**CFU Frequency**	**SE Range in CFU Frequency**
Primary IHBD	BD2	1/22	1/18 – 1/26
	BD3	1/30	1/25 – 1/36
Expanded IHBD	BD2 (passage 7)	1/18	1/15 – 1/22
	BD# (passage 6)	1/20	1/16 – 1/25

3.3.4 Expanded IHBD cells grow from single cells

Single cell derived clones were generated from expanded EpCAM+ IHBD cells in two separate experiments. The frequency of clone formation in the first experiment was 1 out of 40 (Figure 15), indicating a similar colony forming ability observed with total EpCAM+ IHBD cells (Table 7).

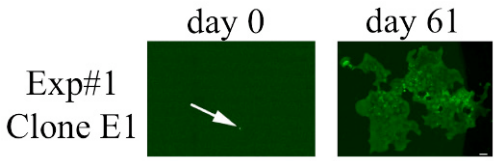


Figure 15: Clonal expansion of EpCAM+ IHBD cells

Single cell self-renewal of was assessed in two experiments. In the first experiment, single expanded EpCAM+ IHBD cells were sorted on one 96-black bottom well plate pre-seeded with rat feeder cells. 40 positive wells, i.e. containing 1 cell were scored. Representative images of a single clone (Clone E1) from this experiment are shown.

3.3.5 Expanded IHBD cells differentiate into bile duct-like structures

Matrigel has been shown to promote or maintain differentiation of both cell lines and primary cells including hepatocytes (139), as well as induce bile duct differentiation of the bipotent hepatoblast cell line, HPPL (107). In addition, we have previously used a matrigel based assay to evaluate the differentiation of mouse gallbladder epithelial cells (135). Therefore, we tested the ability of expanded IHBD cells to differentiate into bile ducts in matrigel. Briefly, IHBD cells were mixed with serum-free media, plated on tissue culture plastic and layered above with

matrigel (Figure 16A). Within one week, small three-dimensional cyst-like structures, similar to those obtained with mouse gallbladder cells, were observed developing in the matrigel, and persisted for at least five weeks in culture (Figure 16A). The cysts were suspended in the matrigel, were hollow (Figure 16B i) and appeared to consist of a single lining of cuboidal epithelial cells (Figure 16B ii). To evaluate the polarization of the epithelial cells lining the cyst lumen, we compared their ultrastructure to those of primary and expanded IHBD cells. TEM analyses indicated that primary and expanded IHBD cells are cuboidal with defined apical-basolateral polarity. They contained of cerebroform nuclei, and exhibited apical microvilli, junctional apparatus and interdigitating lateral membranes typical of bile duct cells (Figure 16C). The cysts forming in the matrigel had a similar ultrastructure, in that they consisted of cuboidal cells with apical microvilli that lining the cyst lumen reminiscent of the cell morphology observed in primary bile ducts.

A key physiological function of primary IHBD cells is to modify biliary content, specifically by secreting water and bicarbonate ions into the bile (7), which is in part mediated by MDR proteins. Rhodamine 123, a fluorescent substrate of MDR1 has been shown to accumulate in the lumens of cysts formed by a hepatoblast cell line grown in matrigel, indicating bile duct differentiation (107). We observed similar transport of the dye by cysts formed from mouse gallbladder stem cells (135). We therefore reasoned that this assay would be an appropriate bile duct differentiation assay for the expanded EpCAM+ IHBD cells. Rhodamine 123 was added to the media of the matrigel cultures and confocal images were taken at various time points. We observed a steady accumulation of the dye in the cyst lumen over the course of an hour (Figure 16D). Importantly, this transport was blocked by addition of Verapamil, an MDR-specific inhibitor (Figure 3D).

Overall these data indicate that the EpCAM+ IHBD cells can differentiate to form bile duct structures *in vitro*, and recapitulate the morphology, ultrastructure and a transport function of primary bile ducts.

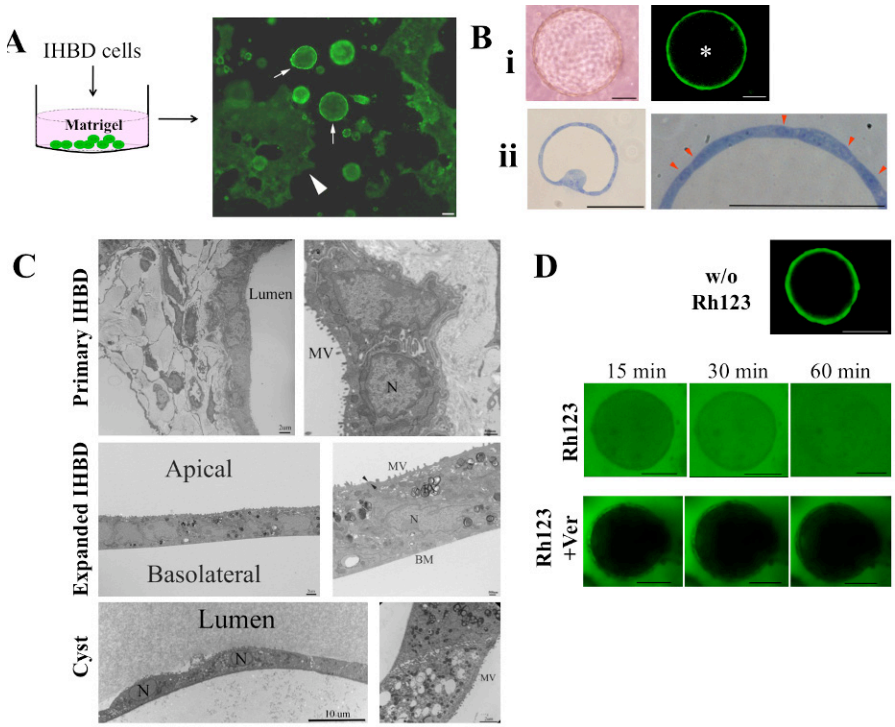


Figure 16: EpCAM+ IHBD cells form bile ducts in vitro

(A) Schematic of differentiation assay. Expanded EpCAM+ IHBD cells (>passage 2) were plated on tissue culture treated dishes in serum-free media and layered with matrigel. Colonies of cells were observed growing on the dish plastic (arrowheads) and cysts were seen growing in the matrigel (arrows). (B). Cysts are hollow and lined by cuboidal cells (i) Brightfield and confocal micrographs of cysts. (ii) Cysts were isolated from matrigel and stained with toluidine blue. (C) Electron micrographs of cysts (31 days post plating), primary IHBD and expanded IHBD cells. Pictures in the right column are higher magnification views of IHBD epithelial cells showing apical-basolateral polarity and interdigitating lateral membranes. (D) Cysts were incubated with 100μM Rhodamine 123 (Rh 123) ± 10μM Verapamil. Confocal micrographs

showing time-lapse images of optical sections corresponding to the center of the cysts at indicated times. Rh 123 accumulates in lumen over 1 hr. Transport is blocked with Verapamil. MV: Microvilli, M: Mitochondria, N: Nucleus, LY: Lysossome, BM: Basement membrane. Unless specified otherwise, scale bars: 100μm.

3.3.6 Expanded EpCAM+ IHBD cells do not differentiate into hepatocytes in vivo

The liver is composed of two epithelial cell types: hepatocytes and IHBD cells. Accordingly, studies involving liver stem cells assess the ability of these cells to differentiate into both cell types. However, there are no definitive *in vitro* hepatocyte differentiation protocols. The *Fah*$^{-/-}$ mouse is an inducible mouse model of liver disease that provides a strong selective advantage for engraftment to donor hepatocytes or hepatocyte-like cells (88, 89). Therefore, we evaluated the ability of expanded EpCAM+ IHBD cells to differentiate into hepatocytes *de novo*, by transplantation into *Fah*$^{-/-}$ mice (Figure 17).

In the first set of experiments, we injected expanded gfp+ EpCAM+ IHBD cells alone into *Fah*$^{-/-}$ mice (Figure 17A). However, after several rounds of selection without NTBC we were not able to detect any engraftment (visualized by gfp+ expression). Expanded gfp+ EpCAM+ IHBD cells were pre-mixed with gfp- primary hepatocytes and injected into *Fah*$^{-/-}$ mice (Figure 17B). Our rationale here is that the donor gfp- hepatocytes would repopulate the liver thereby making the environment more hospitable for EpCAM+ IHBD cells. Therefore, the goal of this experiment was to determine if any EpCAM+ IHBD cells could differentiate into hepatocytes *de novo* and engraft, but not rescue in the liver. In both experiments conducted in this manner, we observed the weight of the animal increasing towards the end of the selection, indicating hepatocyte engraftment and rescue of liver function (Figure 17B). However, we were

not able to detect gfp+ expression in any of the mice. Therefore, based on these data we conclude that expanded EpCAM+ IHBD cells are not capable of *de novo* hepatocyte differentiation in the *Fah*$^{-/-}$ mouse.

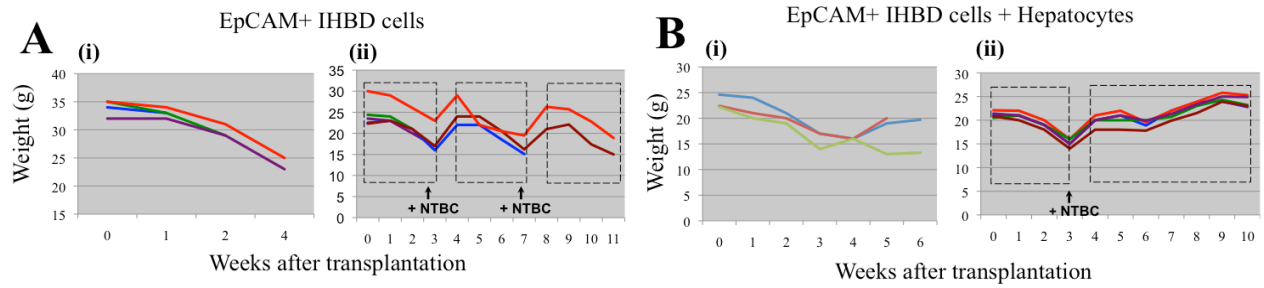


Figure 17: Expanded EpCAM+ IHBD cells do not differentiate into hepatocytes in vivo

(A) 1^{e}6 expanded gfp+ IHBD cells were injected intrasplenically into *Fah*$^{-/-}$ mice. (i) gfp+ IHBD (passage 9) were injected (n=3). Mice were taken off NTBC to induce liver failure over four weeks. (ii) gfp+ IHBD cells (passage 6) were injected (n=5). Three selections without NTBC were performed. (B) 0.4^{e}6 each of expanded gfp+ IHBD cells + gfp- primary hepatocytes were injected intrasplenically into *Fah*$^{-/-}$ mice. (i) One selection without NTCB was performed (n=3). (ii) Two selections without NTBC were performed (n=5). We did not observe gfp+ engraftment in any of the mice injected.

3.4 CONCLUSIONS & FUTURE DIRECTIONS

The identification of resident stem cells in the uninjured liver has been controversial. There is a lack of cell surface markers for the prospective isolation of liver stem cells and their differentiation into hepatocytes has been inconclusive. However, the Canals of Hering have long been implicated as the candidate stem cell niche in the liver (61, 62, 140) indicating that some of all of the IHBD cells might represent liver stem cells. Therefore, in this study we attempt to elucidate the stem cell properties of IHBD cells in the normal uninjured liver, using assays previously developed for the analysis of mouse gallbladder stem cells (135).

Using a combination of MACS and a culture system that selects for epithelial cells, we obtained a pure (>98%) population of IHBD epithelial cells *in vitro*. Most expanded IHBD cells were EpCAM+ and CK19+, two standard markers for bile duct cells (114, 138) and did not express lineage markers CD31, CD45 and F4/80. LDAs performed from primary and expanded EpCAM+ IHBD cells indicated that only a subpopulation of these cells had colony forming ability. However, we were not able to identify a cell surface marker that selected or enriched for this subpopulation. Notably, Dorrell et al. (72) report a very similar CFU frequency (1 out of 34.3) in their characterization of stem cells in the normal liver, suggesting a similar expansion of cells *in vitro*.

Stem cells are defined by their ability to self-renew from single cells and differentiate down lineage-specific programs (91). We demonstrated that expanded EpCAM+ IHBD cells are capable of single cell self-renewal and lineage commitment. Interestingly, the frequency of clone formation was similar to the CFU frequency from the LDA (compare Figure 15 and Table 7). These data suggest that the subpopulation of EpCAM+ IHBD cells capable of colony formation identified by the LDA is also capable of self-renewal from single cells. Clone E1 is

being expanded further and additional clones will be generated. Additional single cell experiments have been performed and are awaiting analysis. The goal is to generate three or four clonal IHBD cultures similar to our experiments with mouse gallbladder stem cells (135). In the future, the phenotypic profiles and morphogenesis of the clonal IHBD cultures will be determined and compared to parent IHBD cultures. In addition, microarray analysis could be used in order to identify specific cell surface markers that enrich for the specific subpopulation of EpCAM+ IHBD cells capable of colony formation similar to a previous study with fetal mouse liver stem cells (141). Last, specific antibodies could be generated against EpCAM+ IHBD cells, similar to a previous study by Dorrell et al. (74), which might allow for the identification of heterogeneous subpopulations within EpCAM+ IHBD cells.

Expanded EpCAM+ IHBD cells exhibited cyst formation in matrigel culture. We found that the cysts recapitulate the ultrastructure and a transport function of primary IHBD cells. Using the matrigel assay, we have characterized cyst formation from adult mouse gallbladder stem cells (135). In addition, Tanimizu et al. (107) found that cyst formation in matrigel by the bipotent hepatoblast cell line –HPPL- was indicative of bile duct differentiation, further corroborating our results. However, to our knowledge cysts formation by primary IHBD epithelial cells have heretofore not been characterized, making our results especially significant. In the future, the expression of EpCAM, CK19, albumin and AAT in cysts will be evaluated, to better elucidate the differentiation status of the cysts.

Last, we determined that expanded EpCAM+ IHBD cells were not able to differentiate into hepatocytes *de novo* in the *Fah*$^{-/-}$ mouse. However, it is possible that *in vitro* expansion makes IHBD cells less amenable to engraftment *in vivo*. Therefore, in the future primary EpCAM+ IHBD cells will be injected with and without primary hepatocytes into *Fah*$^{-/-}$ mice

(n=5/experiment). These experiments have been performed and are awaiting analysis. We predict that primary EpCAM+ IHBD cells will also not be capable of *de novo* differentiation into hepatocytes. One possible explanation for the inability of IHBD cells to differentiate into hepatocytes might be the *Fah*$^{-/-}$ animal model. Using a fate tracing model that specifically labels hepatocytes, Malato et al. (60) have shown that bile duct cells can generate hepatocytes in chronic but not acute models of liver injury. These data reinforce the notion that liver stem cells only play a significant role in tissue repair when the ability of hepatocytes to proliferate is blocked or seriously impaired (58). *Fah*$^{-/-}$ mice while providing a competitive environment for donor hepatocytes to engraft, do not represent a chronic liver injury model that might allow for the *de novo* differentiation of IHBD stem cells into hepatocytes.

Another possible explanation for inability of IHBD cells to differentiate into hepatocytes might be the purity of the donor cells injected. Previous studies reporting the identification of liver stem cells do not clearly preclude the presence of hepatocytes in the donor cell preparation. IHBD cells are a rare population in the small cell fraction from primary liver isolate (Figure 11A). Therefore, obtaining a pure (>98%) primary IHBD cell fraction can be technically challenging. In our experiments for the injection of primary EpCAM+ IHBD epithelial cells into *Fah*$^{-/-}$ mice we found that MACS followed by FACS was essential to obtaining such a pure population. The lack of such a purification protocol could explain how Qiu et al. (70) and Dorrell et al. (72) reported hepatocyte differentiation of liver stem cells in *Fah*$^{-/-}$ mice.

Finally, we evaluated the possibility that EpCAM+ IHBD cells might themselves represent a subpopulation of total bile duct cells. We performed intracellular flow cytometry staining with CK19 and EpCAM. In two separate experiments we found that EpCAM marked a distinct subpopulation of CK19+ cells (Figure 18). Frequency of EpCAM+CK19+ cells varied

between 85-90% of total CK19+ cells depending on the experiment. Therefore, EpCAM+ IHBD cells mark a subpopulation of total bile duct cells. These cells can expand *in vitro*, self-renew from single cells and differentiate to form bile duct structures. However, they are unipotent and are not able to generate hepatocytes *de novo* in *Fah*$^{-/-}$ mice.

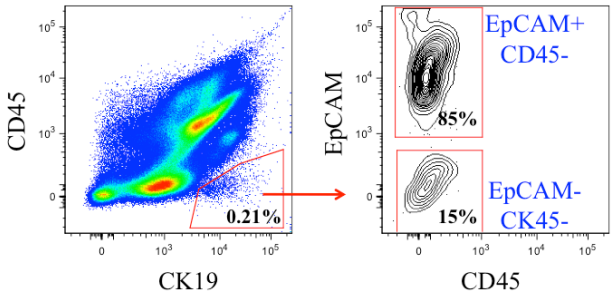


Figure 18: EpCAM marks a subpopulation of primary IHBD cells

Small cell fractions of primary from liver perfusion were stained with EpCAM and CD45, fixed in 4% PFA, permeabilized in 0.1% Triton-X and stained with CK19 (kind gift of Dr. Markus Grompe (Portland, OR). These data indicate presence of a clear population of EpCAM-CK19+CD45- cells (15%). The frequency of this population varied between 10-15% depending on the experiment. Similar results were obtained with Saponin. EpCAM- cells from small cell fractions do not grow on rat feeder cells.

4.0 ISOLATION AND EXPANSION OF A UNIQUE STEM CELL POPULATION FROM FETAL HUMAN GALLBLADDER

4.1 INTRODUCTION

Understanding the resident stem cell populations of the bile duct system is important for both basic biology and designing therapeutic strategies to treat bile duct diseases. The bile duct system is divided into IHBD and EHBD systems, and the EHBD system consists of the common hepatic duct, gallbladder, cystic duct and CBD (2, 142). The gallbladder stores the bile while modifying its content and concentration.

There is a paucity of data characterizing stem cells in the adult or fetal gallbladders, especially in humans. Moreover, the differences between IHBD and EHBD cells are not well understood. The EHBD system, liver and the ventral pancreas develop from the posterior ventral foregut endoderm (9-11). However, the exact mechanisms of development and the cell-intrinsic factors responsible for IHBD and EHBD system specification have heretofore been unclear. It has recently been shown that the IHBD and EHBD system descend from separate progenitors (20). Using a PDX1-Cre mouse, Spence et al. (20) demonstrate that hepatocytes and IHBD cells derive from Pdx1- cells while the EHBD cells and ventral pancreas derive from Pdx1+ cells. These data were corroborated by a study in our lab, where we found that adult mouse gallbladder cells have a distinct phenotypic and expression profile compared to IHBD

cells (135). However, the differences between human IHBD and EHBD cells have not yet been explored.

The goal of this study was to isolate and characterize a resident stem cell population in the fetal human gallbladder and compare its phenotypic and expression profile to those of fetal IHBD cells. The evaluation of human fetal gallbladder stem cells could have important ramifications for the study of congenital diseases such as biliary atresia (21) and would also help elucidate the ontogeny of cells in the bile duct system.

Stem cells are defined by their ability for single cell self-renewal and lineage commitment (91). In a previous study, we have used colony forming assays along with single cell and morphogenesis assays to characterize a resident stem cell population in adult mouse gallbladder (135). In this report, we will adapt the assays to human cells in order to isolate and characterize resident stem cells in the fetal human gallbladder.

4.2 MATERIALS AND METHODS

4.2.1 Gallbladder and IHBD cell isolation and culture

Fetal liver and gallbladder tissues were obtained from the Tissue Bank at the Magee Women's Hospital of UPMC. All samples were between 19-23 weeks of gestation. The research protocol was reviewed and approved by the Institutional Review Board for Human Research Studies at the University of Pittsburgh. Gallbladders were cut and opened along the middle in order to expose the mucosa. Bile was washed off my gently scraping the mucosal surface with blunt forceps in HBSS. Liver samples were minced into small pieces. Samples were incubated with

EBSS/10mM EGTA/1% HEPES for 15min and treated with 1 mg/ml CollagenaseII (Invitrogen, CA) +1mg/ml Hyaluronidase (Sigma) + 100 μg/ml of DNaseI (Roche, IN) for 1 hour followed by 0.25%Trypsin /0.1%EDTA (Fisher Scientific, MA) for 30 min to obtain a cell suspension. Cell suspensions were plated on irradiated rat feeder cells as described previously (135).

4.2.2 Immunofluorescence

Antibodies against EpCAM (BD Biosciences, Dilution 1:100), CK19 (Santa Cruz Biotechnology, Dilution 1:300) and AAT (Bethyl Laboratories, Dilution 1:50) were used in PBS. Sections were blocked with 0.5% milk and stained with either anti-rat or anti-rabbit Alexa Fluor® secondaries (Invitrogen, CA). Images were taken using an IX71 Inverted microscope (Olympus, PA).

4.2.3 FACS Analysis

FACS analysis and sorting and subsequent data analysis was performed as previously described (135).

4.2.4 Collagen differentiation assay

Gallbladder cells were plated in tissue culture dishes at medium density (50,000- 100,000 cells/well of 48 well plate) and layered above with an equal volume of PureCol® (Advanced Biomatrix) (PureCol® is a version of bovine dermal hide collagen or Vitrogen) and placed at

37C for 30min. The gels were covered with DMEM/F12 and 1% ITS and recombinant human EGF (50ng/ml) (Peprotech). Media was changed twice a week.

4.3 RESULTS

4.3.1 EpCAM is a human fetal gallbladder epithelial cell marker

EpCAM is a cell surface marker that was first described in colorectal cancer (143). Its expression has since been found on a wide variety of epithelial cells such as keratinocytes, thymic epithelial cells and intrahepatic bile duct (IHBD) cells (114, 144). In our previous report on adult mouse gallbladder stem cells, we identified that gallbladder epithelial cells were EpCAM+, and subsequently used EpCAM to label these cells by flow cytometry (135). EpCAM expression has also been observed on adult human gallbladder epithelial cells (129, 145) but no evidence exists for its expression in fetal gallbladder. We performed co-localization studies with EpCAM and CK19, a pan biliary marker (115). We found that most CK19+ cells were EpCAM+ (Figure 19A). Therefore, EpCAM marks human fetal gallbladder epithelial cells.

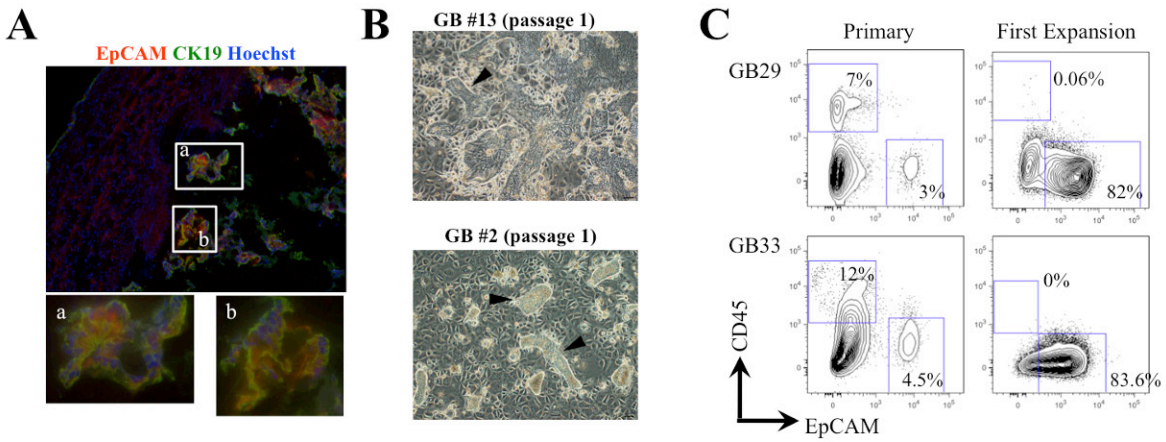


Figure 19: Fetal human gallbladder epithelial cells expand on feeder cells

A) Sections of human fetal gallbladder were stained with EpCAM (red) and CK19 (green). Most CK19+cells were EpCAM+ (yellow staining). (B) Fetal human gallbladder cells were isolated and expanded *in vitro* on lethally irradiated LA7 feeder cells. Representative pictures of two human samples indicating epithelial expansion (arrowheads). (C) *In vitro* growth conditions select for gallbladder epithelial (CD45-EpCAM+) cells. Flow cytometric analyses at primary and first expansion (p0) of two gallbladder samples indicating strong enrichment of CD45-EpCAM+ cells.

4.3.2 Human fetal gallbladder epithelial cells expand in vitro

Gallbladder cells were cultured *in vitro* on lethally irradiated rat feeder cells that select for epithelial growth (95), similar to our previous study on mouse gallbladder cells (135). In total 23 fetal gallbladder samples were processed. All samples that were put into culture (n=21) exhibited *in vitro* expansion (Figure 19B). Small cells growing in cobblestone-like epithelial colonies were the hallmark of these cultures along with colonies comprising small cells with large nuclear to cytoplasmic ratios. Flow cytometry analyses of primary and expanded

gallbladder cells at first expansion (p0) showed that feeder cells strongly select for epithelial (EpCAM+) expansion (Figure 19C). EpCAM- cells that were sorted from primary gallbladder did not proliferate. Similar to previous studies (129, 146), we observed that LA7 feeder cells allowed for up to 7-8 serial passages of gallbladder cells after which these cells exhibited irreversible growth arrest.

4.3.3 CD13 and CD44 are heterogeneously expressed on primary gallbladder cells

Since there is a paucity of cell surface markers for human gallbladder stem cells, we began by screening primary and expanded cells with a panel of 44 commercially available cell surface markers of stem and progenitor cells (Table 8). As stem cells have the capacity for self-renewal, we predicted that expansion *in vitro* would select for cells capable of self-renewal, thereby enriching for primitive or stem cells. Our goal here was to find markers that were heterogeneously expressed on primary gallbladder epithelial (EpCAM+) cells and were enriched on expanded gallbladder cells. In this manner, we hoped to identify candidate stem cell markers.

Five separate expanded human gallbladder samples were screened with the foregoing panel of cell surface markers. We found that phenotypic expression of expanded gallbladder cells was remarkably conserved in culture suggesting expansion of a homogeneous subpopulation of cells (Table 8). Conversely, the phenotypic expression of primary gallbladder cells (n=5) was variable indicating the potential difference between separate biological samples (Table 8). Of the various markers, CD13 and CD44 consistently showed heterogeneous expression on primary gallbladder epithelial cells (Table 8 and Figure 20A). CD13 (aminopeptidase N) has previously been described as a stem cell marker in the developing

mouse liver (147, 148) as well in human hepatocellular carcinoma (149). In addition, recent studies have indicated that CD44 (the receptor for hyaluornic acid) has recently been shown to a human gallbladder cancer stem cell marker (46, 47). Interestingly, co-staining with CD13 and CD44 showed additional heterogeneity in the primary gallbladder epithelial cells (Figure 20B). We noted the presence of three distinct subpopulations of epithelial (EpCAM+) cells: CD44+CD13+ cells, CD44-CD13+ and CD44-CD13- cells. The CD44-CD13+ subpopulation was by far the largest in all samples analyzed (n=9), followed by the CD44+CD13+ subpopulation. Expanded gallbladder cells were CD44+ and heterogeneous for CD13 expression (Figure 20B). We observed the presence of two distinct subpopulations of expanded gallbladder epithelial (EpCAM+) cells: CD44+CD13+ and CD44+CD13- cells. Interestingly, when we analyzed the same gallbladder samples from primary tissue and at first expansion (p0), we observed that the frequency of CD44+CD13+ cells remained relatively constant (Figure 20C). Based on these data, we hypothesized that the CD44+CD13+ subpopulation in primary gallbladder epithelium represented cells capable of self-renewal and would be the most highly enriched for primitive or stem cells.

Table 8: Phenotypic profiles of primary and expanded human gallbladder cells

Antibody	**Fetal GB2** (p4)	**Fetal GB8** (p1)	**Fetal GB13** (p2)	**Fetal GB24** (p1)	**Fetal GB26** (p2)
EpCAM	+	+	+	+	+
CD13	Het	Het	Het	Het	Het
CD44	+/Het	+	+	+	+
CD49b	+	+	+	+	+
CD49f	+	+	+	+	+
CD54	+/Het	+/Het	+/Het	+/Het	+/Het
CD227	-	-	-	-	-
CD66	+/Het	+/Het	+/Het	+/Het	+/Het
CD49d	-	-	-	-	-
CD106	-	-	-	-	-
MDR	-	-	-	-	-
CD117	-	-	-	-	-
MRP1	-	-	-	-	-
CD49a	-	-	-	-	-
CD133.1	+	+	+	+	+
CD9	-	-	-	-	-
CD133.2	+	+/Het	+	+	+
CD90	-	-	-	-	-
CD64	-	-	-	-	-
CD49c	+/low	+	+	-	+
CD29	+	+	+	+	+
CD20	-		-	-	-
CD166	+	+	+	+	+
CD81	+/het	+	+	+	+
E-cadherin	-	-	-	-	-
CD104	+/low	+/low	+/low	+/low	+/low
CD71	-	-	-	-	-
CD34	-	-	-	-	-
CD73	-	-	-/Het?	-	-
CD36	-	-	-	-	-
CD24	-	-	+	-	+
CD140b	-	-	-	-	-
CXCR4	-	-	-	-	-
CD45	-	-	-	-	-
CD130	-	-	-	-	-
CD26	-	-	-	-	-
CD3	-	-	-	-	-
CD1a		-	-	-	-
CD49e				-/low	-
CD56				-	-
CD38			-	-	-
CD80			-	-	-
EGFR			-	-	-
EphB2			-	-	-
CD11b				-	-

Antibody	**Fetal GB11 Primary**	**Fetal GB13 Primary**	**Fetal GB24 Primary**	**Fetal GB22 Primary**
EpCAM	+	+	+	+
CD13	Het	Het	Het	Het
CD44	Het	Het	+/Het	-
CD49b	+	+	+/Het	+
CD49f	+	+	+/Het	+/Het
CD54	-/Het	-	+/Het	-
CD227	-		-	-
CD66	-	-	-	-
CD49d	-	-	-	-
CD106			-	-
MDR			-	-
CD117			-	-
MRP1			-	-
CD49a			-	-
CD133.1	+	+	-	-
CD9			-	-
CD133.2		+	-	-
CD90			Het	Het
CD64			-	-
CD49c			-	-
CD29	+/Het	+	+	+/Het
CD20			-	-
CD166	+/Het	+	-	+/low
CD81	Het	Het	Het	-
E-cadherin			-	-
CD104			-	-
CD71			-	-
CD34				-
CD73	+/Het	-/low	-	-
CD36			-	-
CD24			-	-
CD140b			-	-
CXCR4			-	-
CD45			-	-
CD130			-	-
CD26	+		-	-
CD3			-	-
CD1a			-	-
CD49e		-		
CD56	-	-		
CD38			-	-
CD80			-	-
EGFR			-	-
EphB2			-	-
CD11b				

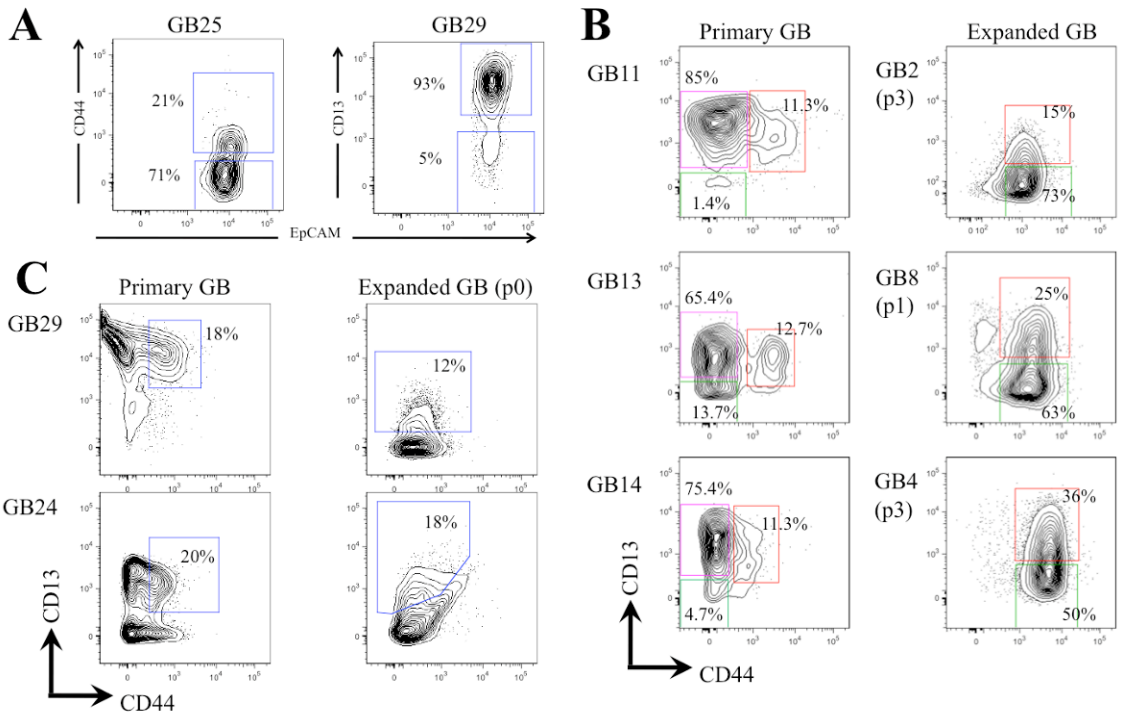


Figure 20: EpCAM+CD13+CD44+ cells are enriched with in vitro expansion

A) Flow cytometric profile showing that CD13 and CD44 are heterogeneous in primary human fetal gallbladder epithelial cells. Flow plots represent only EpCAM+ cells from primary gallbladder. EpCAM- cells have been gated out. (B) Co-expression of CD13 and CD44 identifies three subpopulations in primary human fetal gallbladder epithelial cells (left panel). These subpopulations are: CD13+CD44+, CD44-CD13+ & CD13-CD44- cells. Only CD13 was heterogeneous in expanded gallbladder epithelial cells (right panel). Rat feeder cells have been gated out in these flow plots. (C) Phenotypic profiles of CD13 and CD44 on the same gallbladder sample from primary tissue (d0) or in *in vitro* culture. These data suggest that CD44+CD13+ cells are enriched *in vitro* and represent the clonogenic fraction in primary gallbladder.

4.3.4 CD44 and CD13 enrich for human gallbladder stem cells

In order to evaluate CD13 and CD44 as gallbladder stem cell markers, we performed LDAs. The LDA assay quantifies the frequency of a specific subpopulation of cells with a biological activity (99) and was key to the isolation of hematopoietic (100) and neural stem cells (101). In the evaluation of stem cells, biological activity is typically defined as the ability to form a colony and the LDA serves to quantify the frequency of stem and progenitor cells.

We first determined if CD13 and CD44 could independently enrich for self-renewal. We separated EpCAM+CD13+ and EpCAM+CD13- cells from primary gallbladders and performed LDAs with the sorted cells. EpCAM+CD13+ cells exhibited a significantly higher CFU frequency (1/28) than EpCAM+CD13- cells (1/195) (Table 9A). In a separate experiment, we separated EpCAM+CD44+ and EpCAM+CD44- cells from primary gallbladders and performed LDAs with the sorted cells. EpCAM+CD13+ cells exhibited a significantly higher CFU frequency (1/17) than EpCAM+CD13- cells (1/66) (Table 9A). Therefore, both CD13 and CD44 independently enrich for colony forming cells confirming that they are gallbladder stem cell markers.

We then determined if combined expression of CD13 and CD44 further enriched for gallbladder stem cells. In two separate experiments, EpCAM+CD44+CD13+, EpCAM+CD44-CD13+ and EpCAM+CD44-CD13- subpopulations of cells were sorted from primary gallbladders (Table 9B). In both experiments, we noted that the EpCAM+CD44+CD13+ cells exhibited the highest colony forming cells (ranging from 1/8 to 1/39) and the EpCAM+CD44-CD13- cells the lowest (1/134 to 1/664). EpCAM+CD44-CD13+ cells had the same approximate CFU frequency as the bulk epithelial (EpCAM+) cells. Interestingly, in both experiments EpCAM+CD44+CD13+ cells had a significantly higher CFU frequency than the

bulk epithelial cells. These data indicate that combined expression of EpCAM, CD44 and CD13 significantly enriches for gallbladder epithelial stem cells, and represent the cells that expand *in vitro*.

Table 9: Co-expression of CD13 and CD44 enriches for gallbladder epithelial stem cells

LDAs from primary gallbladder were performed as described before (135). (A) EpCAM+CD44+/- and EpCAM+ CD13+/- epithelial cells were sorted to independently confirm enrichment in self-renewal. CFU frequency ±SE (L-Calc®) indicates that CD13 and CD44 independently enrich for stem cells. (B) Combined expression of CD13 and CD44 was used to further enrich for self-renewal. EpCAM+CD13+CD44+, EpCAM+CD44-CD13+ & EpCAM+CD13-CD44- cells were sorted in two different experiments. CFU frequency ±SE (L-Calc®) indicates that EpCAM+CD44+CD13+ cells are the most enriched in self-renewal.

Sample	Sorted Population	CFU Freq	SE in CFU Freq
A			
GB25 (Primary Cells d0)	EpCAM+	1/38	1/27 – 1/54
	EpCAM+CD44+	1/17	1/12 – 1/25
	EpCAM+CD44-	1/66	1/47 – 1/94
GB29 (Primary Cells d0)	EpCAM+	1/24	1/17 – 1/33
	EpCAM+CD13+	1/28	1/21 – 1/39
	EpCAM+CD13-	1/195	1/125 – 1/303
B			
GB14 (Primary Cells d0)	EpCAM+	1/309	1/247 – 1/388
	EpCAM+CD44+CD13+	1/39	1/28 – 1/56
	EpCAM+CD44-CD13-	1/664	1/428 – 1/1,030
	EpCAM+CD44-CD13+	1/418	1/285 – 1/614
GB31 (Primary Cells d0)	EpCAM+	1/22	1/16 – 1/30
	EpCAM+CD44+CD13+	1/8	1/5 – 1/13
	EpCAM+CD44-CD13-	1/134	1/65 – 1/274
	EpCAM+CD44-CD13+	1/23	1/16 – 1/33

To further confirm these data, we performed LDAs from expanded gallbladder cells. The rationale is that EpCAM+CD44+CD13+ cells from primary gallbladder expand *in vitro*. Therefore, EpCAM+CD44+CD13+ cells from expanded gallbladder should also represent the colony forming cells giving further proof to the fact that CD44 and CD13 are gallbladder stem cells. In these studies, EpCAM+CD44+CD13+ cells were separated from EpCAM+CD44+CD13- cells in four different expanded human gallbladder samples (Table 10). In all samples analyzed, we observed that EpCAM+CD44+CD13+ cells had a significantly higher CFU frequency (ranging from 1/18 to 1/39) than EpCAM+CD44+CD13- cells (1/77 to 1/208). However, opposite to what we had observed in primary gallbladder cells, the CFU frequency of EpCAM+CD44+CD13+ cells was not significantly higher than bulk epithelial (EpCAM+) cells in any of the samples (Table 10). These data confirm that CD44 and CD13 are gallbladder stem cell markers, but also suggest that *in vitro* culture selects for a homogeneous expansion of cells. It appears that in order to definitively identify subpopulations of cells that are highly enriched in colony forming ability from the gallbladder combined surface marker expression on primary cells not expanded cells, might be the best strategy.

Table 10: EpCAM+CD44+CD13+ remain the clonogenic fraction in vitro

LDAs were performed from expanded gallbladder. EpCAM+CD44+CD13+ and EpCAM+CD44+CD13- cells were separated in four different gallbladders. CFU frequency ±SE (L-Calc®) indicates that EpCAM+CD44+CD13+ cells are enriched in colony formation compared to EpCAM+CD44+CD13- cells.

Sample	Sorted Population	CFU Freq	SE range in CFU Freq
GB2 (passage 2)	EpCAM+	1/56	1/45 – 1/68
	EpCAM+CD44+CD13+	1/39	1/30 – 1/51
	EpCAM+CD44+CD13-	1/96	1/75 – 1/123
GB2 (passage 1)	EpCAM+	1/26	1/21 – 1/33
	EpCAM+CD44+CD13+	1/21	1/15 – 1/28
	EpCAM+CD44+CD13-	1/208	1/105 – 1/280
GB4 (passage 4)	EpCAM+	1/49	1/40 – 1/60
	EpCAM+CD44+CD13+	1/65	1/51 – 1/83
	EpCAM+CD44+CD13-	1/167	1/129 – 1/215
GB8 (passage 2)	EpCAM+	1/60	1/49 – 1/73
	EpCAM+CD44+CD13+	1/18	1/13 – 1/24
	EpCAM+CD44+CD13-	1/77	1/59 – 1/102
GB24 (passage 1)	EpCAM+	1/27	1/22 – 1/33
	EpCAM+CD44+CD13+	1/19	1/14 – 1/25
	EpCAM+CD44+CD13-	1/84	1/63 – 1/112

4.3.5 Expanded gallbladder cells grow from single cells

In order to determine stemness of gallbladder cells, we first determined if they are capable of single cell or clonal self-renewal. Initial experiments were performed with expanded gallbladder cells, where single EpCAM+ cells were sorted on 384-well plates seeded with LA7 feeder cells and each well was imaged (Figure 21). As the CFU frequency of bulk EpCAM+ cells from expanded gallbladder was not significantly different from that of EpCAM+CD44+CD13+ cells (Table 10), we reasoned that sorting bulk EpCAM+ cells would be an appropriate assay to determine clonal self-renewal. The lipophilic membrane labeling red fluorescent dye PKH26 (150) was added to the cells, in order to visualize them after cells sorting. 60 positive wells were scored in the first experiment and 1 clone was generated (Figure 21). Therefore, expanded gallbladder cells are capable of clonal self-renewal.

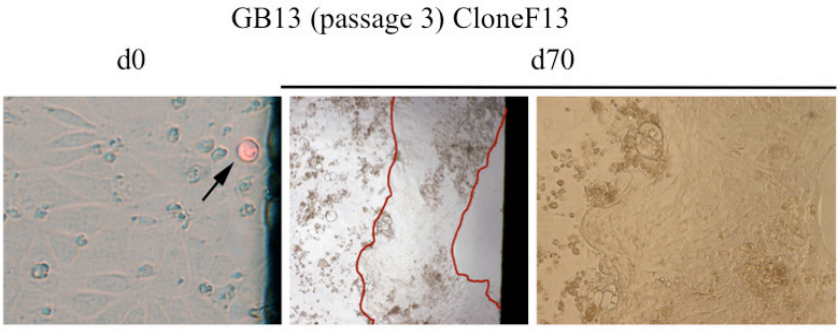


Figure 21: Expanded EpCAM+ gallbladder cells exhibit single cell self-renewal

Preliminary analysis of clonal self-renewal. Single expanded EpCAM+ gallbladder cells were plated on 384-well plates pre-seeded with rat feeder cells. 60 positive wells were scored and a single clone (Clone F13) was generated (imaged at day 70). Scale bars: 100μm.

4.3.6 Expanded gallbladder cells differentiate into gallbladder-like structures in vitro

We developed a novel *in vitro* differentiation assay by utilization the bovine dermal hide collagen, Vitrogen as an extracellular matrix. Collagen supplemented with EGF has been used to observe morphogenesis of rabbit gallbladder epithelial cells (127, 128, 151). However, there are no studies looking at the 3D morphogenesis of human gallbladder epithelial cells. We mixed expanded bulk epithelial (EpCAM+) cells serum free media and layered above with Vitrogen supplemented with EGF (Figure 22A). Within the first week, we observed the formation of cyst like structures (Figure 22B). The cysts were morphologically similar to those observed with rabbit gallbladder (127, 128, 151) and mouse gallbladder cells (135).

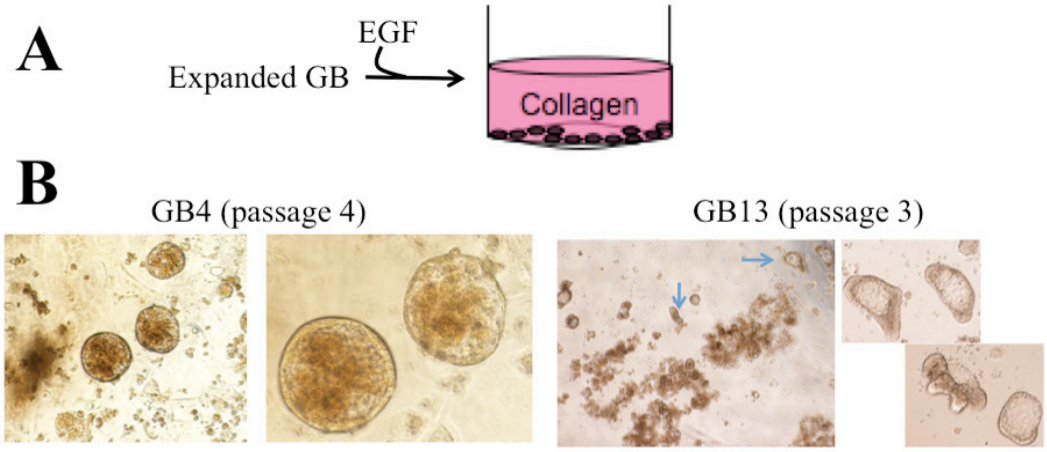


Figure 22: Expanded EpCAM+ gallbladder cells differentiate to form in vitro gallbladders

Preliminary evaluation of gallbladder differentiation. Expanded EpCAM+CD44+CD13+ cells were plated on tissue culture treated dishes in serum-free media and layered with collagen supplemented with EGF (50µg/ml). Representative images of cysts were taken at 21 days post plating. Similar morphogenesis was observed with two additional gallbladder samples. Scale bars: 100µm.

4.3.7 Gallbladder cells are unique compared to IHBD cells

A recent study has shown that IHBD and EHBD systems develop from separate progenitors during mouse development (20). In addition, we have shown that adult mouse gallbladder cells have a distinct phenotypic and expression profile compared to IHBD cells (135). However, there have no reports of the molecular differences –if any- between human gallbladder and IHBD cells. For these reasons, we compared human fetal gallbladder and IHBD cells. We reasoned that as the phenotypic profiles of primary human gallbladder cells were variable (Table 8), similar variability would be obtained with primary IHBD cells. Therefore, we began by expanded human fetal IHBD cells *in vitro* on LA7 feeder cells. Briefly, fetal livers were digested and cell suspensions were grown on lethally irradiated LA7 feeder cells (see *Material and Methods*). We observed that at least two serial passages allowed for expansion of cobblestone-like bile duct colonies comprised of small cells with large nuclear to cytoplasmic ratios (Figure 23A). Expanded IHBD cells were EpCAM+, CD227+ and CK19+ and did not express the hepatocyte marker, alpha-1-antitrypsin (AAT) (Figure 23B). CD227 (Mucin 1) is known to be expressed on human fetal IHBD cells (152). Overall these data show that human fetal IHBD cells can be successfully expanded on LA7 feeder cells.

We screened expanded IHBD cells (n=5) with the same panel of cell surface markers used on gallbladder cells. Similar to the gallbladder cells, we found that various IHBD cell samples had a conserved phenotypic profile *in vitro*. Interestingly, we found that expression of seven cell surface markers, CD13, CD49b, CD54, CD227, CD6, CD104 and CD26, were different between gallbladder and IHBD cells (Figure 23C) suggesting that these cells are distinct from each other.

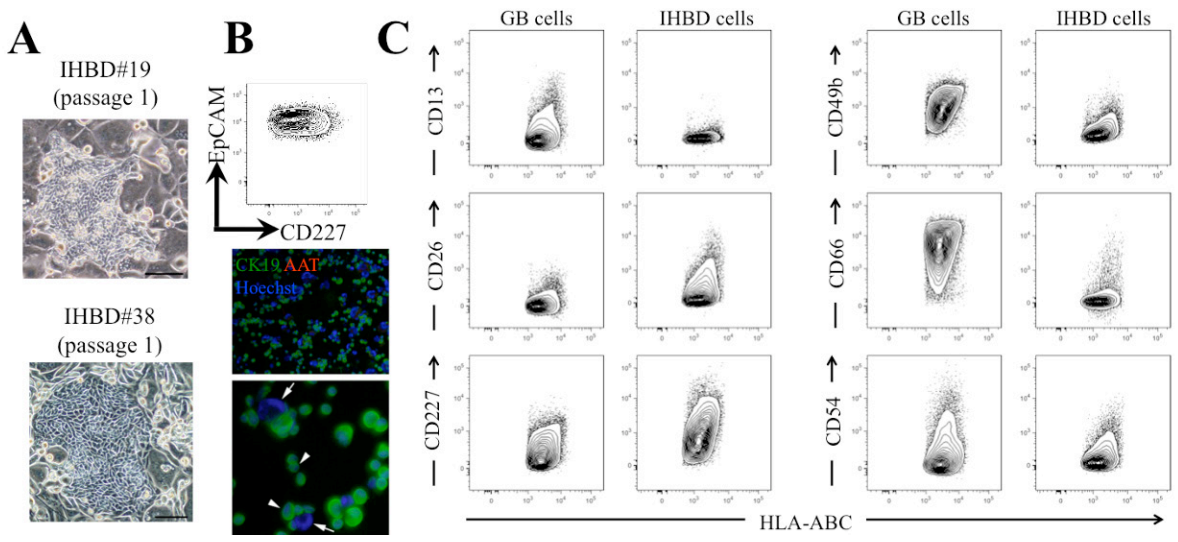


Figure 23: Gallbladder cells have a distinct phenotypic profile compared to IHBD cells

(A) Livers were digested and total cells were plated on rat feeder cells. Following several (at least two passages), we observed a robust expansion of bile duct colonies. Representative pictures of two fetal liver samples at early passage indicating IHBD cell expansion. (B) Flow cytometric profile of IHBD#19 (passage 1) showing that all cells are EpCAM+CD227+. Immunostaining of IHBD#19 to show that all cells were CK19+AAT- (arrowheads; rat feeder cells (arrows). (C) Expanded IHBD cells (n=5) were screened with the same panel of 44 cell surface markers screened on gallbladder cells (n=5) (see Figure 2). Each group (expanded GB or expanded IHBD) exhibited a homogeneous staining pattern, with consistent differences in select markers between each group. We found expression of CD13, CD26, CD227, CD49b, CD66 and CD54 were different between expanded gallbladder and IHBD cells. Scale bars: 100μm.

4.4 CONCLUSIONS AND FUTURE DIRECTIONS

There is currently a paucity of data for the identification of resident stem cells in the uninjured human gallbladder. In addition, the differences between human gallbladder cells and IHBD cells have not been explored. We have previously developed *in vitro* expansion and differentiation assays for the characterization of adult mouse gallbladder stem cells (135). Here we adapt these assays to identify a fetal human gallbladder epithelial or resident stem cell. We found that EpCAM+CD44+CD13+ cells represent the clonogenic subpopulation in primary human fetal gallbladder. These cells can expand *in vitro*, give rise to EpCAM+CD44+CD13- cells, can expand from single cells and differentiate into gallbladder-like structures. These data suggest that EpCAM+CD44+CD13+ fetal gallbladder cells are enriched in stem cells. In addition, preliminary comparison of expanded human gallbladder and IHBD cells indicate that the two cell types have distinct phenotypic profiles *in vitro*.

This study provides compelling data suggesting that EpCAM+CD44+CD13+ cells are enriched in stem cells. In the future, two single cell clonal experiments will be carried out with primary EpCAM+CD44+CD13+ and EpCAM+CD44-CD13+ cells to confirm that only the former subpopulation can expand from single cells. EpCAM+CD44-CD13- cells will not be evaluated in this manner because this subpopulation consistently exhibits the least self-renewal (Table 9) and low cells numbers make this experiment technically challenging. These experiments have been performed and are awaiting analysis. *In vitro* differentiation of human gallbladder cells will be evaluated by comparing ultrastructure of cysts will that of expanded and primary gallbladder cells and by a transport assay previously performed on mouse gallbladder stem cells (135). Finally, the transcriptomes of expanded gallbladder (n=8) and

IHBD cells (n=8) will be compared by oligonucleotide microarray analysis. The samples have already been prepared and submitted for analysis.

This study is to our knowledge, the first to identify resident stem cells in the human fetal gallbladder and to compare these cells to IHBD cells. We have described a novel feeder cell culture system that select for human gallbladder epithelial cells and supports single cell self-renewal. In a previous study, Kobayashi et al. (94) used human gallbladder myofibroblasts as feeders to culture gallbladder cells. However, their culture system does not select for epithelial cells and clonal expansion was not reported.

Using the LA7 feeder cells and the LDA colony forming assay, we were able to determine specific cell surface markers that select for self-renewal in primary gallbladder. We found that EpCAM+CD44+CD13+ cells have a significantly enriched CFU readout than EpCAM+CD44-CD13- cells (~17-fold; Table 9). Interestingly, in two separate experiments we noted that the EpCAM+CD44+CD13+ cells had a significantly higher CFU readout that the bulk epithelial (EpCAM+) cells. These data suggest that combined expression of two markers significantly enriches for stem and/or primitive cells. However, additional makers would be required to prospectively select for the subpopulation can expand robustly from single cells (126, 135).

EpCAM+CD44+CD13+ expanded *in vitro* to generate CD13+ and CD13- cells and retained their higher CFU ability compared to the EpCAM+CD44+CD13- cells (Table 10). However, this CFU ability was not significantly higher than bulk epithelial (EpCAM+) cells in any of the expanded gallbladder samples tested, indicating expansion of a relatively homogeneous population of cells *in vitro*. The phenotypic profiles of primary and expanded gallbladder epithelial cells corroborated these data (Table 8). Primary gallbladder epithelial cells

had a variable phenotypic profile regarding expression of CD166, CD133.1, CD133.2 and CD26. In contrast, expanded cells had a conserved phenotypic profile. In all, these data indicate that expansion *in vitro* does select for primitive or stem cells and suggest that fractionated from the primary gallbladder would be necessary to separate functionally distinct stem and non-stem cell populations. For these same reasons, we compared bulk expanded epithelial (EpCAM+) cells with expanded IHBD cells.

We found that LA7 feeder cells selected for expansion of IHBD epithelial cells at the expense of hepatoblasts and non-epithelial cells over several passages. It is thought that IHBD epithelial cells develop around seven or eight weeks post gestation (12), and hepatoblasts not involved in ductal plate formation are CK19- by around 14 weeks post gestation (153). In addition, by 20 weeks of gestation, IHBD cells are CK19+ and CD227+ (153). All the fetal livers processed were between 19-23 weeks of gestation. Accordingly, we observed that expanded IHBD cells were CK19+, CD227+, AAT- and EpCAM+ (Figure 23). Phenotypic comparison of IHBD and gallbladder cells indicated differential expression of CD13, CD49b, CD54, CD227, CD66 and CD26. These data are especially interesting, as we have identified CD13 as a gallbladder stem cell marker in this study. We are currently evaluating the differences in global gene expression between expanded IHBD and gallbladder cells by oligonucleotide microarrays.

5.0 PRELIMINARY DIFFERENTIATION OF MOUSE GALLBLADDER STEM CELLS INTO HEPATOCYTES

5.1 INTRODUCTION

Liver disease is among the top causes of death in the US (75) affecting roughly 10% of all Americans. In addition, 170 million people worldwide and 4 million in the US are infected with Hepatitis C (76), which is a major cause of liver failure in the US (77). The only widespread current treatment for chronic liver disease is OLT. However, there are over 17,000 people on the waiting liver for a transplant and only 5,000 available donors (78). In addition, OLT is an expensive procedure reducing the number of people with the wherewithal to afford one.

An alternative to OLT is hepatocyte transplantation, which has had some clinical success (79), but is limited by a shortage of donor livers and by the inability of hepatocytes to expand *in vitro*. For these reasons, alternative cell-based therapies are being pursued to treat liver disease. One such alternative is a liver stem cell. However, the identification of a liver stem cell has proved controversial and elusive. In addition, their differentiation into hepatocytes *in vivo* has not been definitively proven. Last, availability of liver stem cells would be hampered by similar limitations that hamper the availability of hepatocytes. This problem of availability can be circumvented by the use of a more readily available tissue stem cell such as a gallbladder stem cell.

The gallbladder is considered to be a non-vital organ and removal of the gallbladder is a routine surgery with 500,000 – 600,000 performed every year (80). The sheer volume of available tissue therefore presents exciting opportunities for candidate cell-based therapy. In addition, the gallbladder and hepatocytes derive from the ventral foregut endoderm (12). Based on this shared ontogeny, we hypothesize that it would be relatively easier to reprogram a gallbladder cell into a hepatocyte, than a fibroblast into a hepatocyte. Moreover, the use of gallbladder stem cells would circumvent some of the major problems reported with the differentiation of induced pluripotent stem (iPS) cells such as rejection by the immune system (154) or the generation of immature hepatocytes (155).

We have previously reported the isolation and expansion of adult mouse gallbladder stem cells (135). We noted the stable long-term (> passage 5) expansion of gallbladder stem cells. In this study, we attempted to obtain preliminary data for the differentiation of the mouse gallbladder stem cells into hepatocytes.

5.2 MATERIALS AND METHODS

5.2.1 Isolation of primary EpCAM+ gallbladder cells

Mouse gallbladder cells were isolated and described previously (135). Total gallbladder cell isolates were stained with EpCAM-PE (BioLegend, Dilution 1:50) primary antibodies followed by anti-PE microbead conjugated secondaries (Miltenyi Biotec) and eluted through a MS MACS® separation column (Miltenyi Biotec). The number of microbead conjugated secondary was adjusted for the low cell frequency of EpCAM+ cells. We did this by recalculating the total

cell number. First we assumed that at least 90% of the actual number of total cells were EpCAM+ cells and this number was 5% of the recalculated total cells. We then adjusted the volume of microbeads according to this recalculated total cell number. MACS+ fractions were snap frozen for gene array.

5.2.2 Isolation of primary hepatocytes

Hepatocytes were harvested using the 2-step collagenase perfusion technique introduced by Seglen et al. (109). Following isolation, hepatocytes were separated by a low speed centrifugation (50g, 2min). The number and viability of cells were determined by trypan blue exclusion. Cells were snap frozen for gene array.

5.2.3 In vitro differentiation of gallbladder cells into hepatocyte-like cells

Expanded EpCAM+CD49f+ cells were grown in sandwich culture between collagen and matrigel. Media was supplemented with HGF (40ng/ml), EGF (25ng/ml) or Dex (100nM) as previously described (81). The cells were harvested after two weeks.

5.2.4 Oligonucleotide Microarrays

Oligonucleotide microarrays were performed as previously described (135). Final Analysis was performed on raw data normalized to median value for each array followed by combat normalization to remove batch effects (110). Hierarchical clustering was performed in BRB-ArrayTools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team by

centered correlation and average linkage methods. Significant transcripts were defined in BRB-ArrayTools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team ($p<0.001$). Differentially expressed genes were annotated and analyzed in Ingenuity Pathway Analysis (Ingenuity® Systems CA, www.ingenuity.com). Heatmaps were generated in Partek® software, version 6.5 Copyright© 2010 Partek Inc., St. Louis, MO, USA. Venn Diagrams were generated in the online tool http://www.pangloss.com/seidel/Protocols/venn.cgi

5.3 RESULTS

5.3.1 Hepatic differentiation by in vitro culture conditions

Kuver et al. have recently reported the differentiation of mouse gallbladder epithelial cells into hepatocyte-like cells *in vitro* (81). To establish a proof-of-concept, we repeated their experiments with the adult mouse gallbladder stem cells described previously (135). Similar to their protocol, EpCAM+CD49f+ mouse gallbladder cells were grown in sandwich culture between collagen and matrigel in the presence of HGF, EGF and Dex (Figure 24A). RT-PCR analysis showed that in the combined presence of HGF, EGF and Dex, gallbladder cells grown in this manner expression albumin (Figure 24B). Importantly, these cells did not expression albumin prior to differentiation or with HGF, EGF or Dex alone. These data establish the feasibility of hepatic differentiation from gallbladder stem cells and merit further investigation. Subsequent analysis on the differentiated cells showed that all gallbladder cells before and after differentiation expressed CK19. Therefore, in current in vitro conditions, mouse gallbladder stem cells differentiate into hepatocyte-like cells, but not mature hepatocytes. These data

suggest that a more direct approach of reprogramming with specific transcription factors might be necessary.

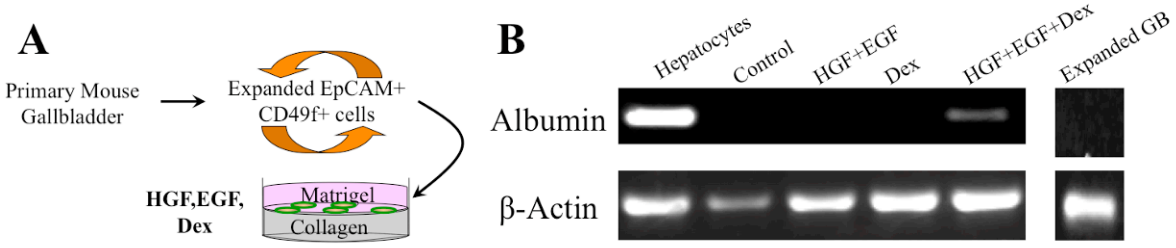


Figure 24: Hepatic differentiation of expanded EpCAM+CD49f+ gallbladder stem cells by culture conditions

(A) EpCAM+CD49f+ gallbladder stem cells were expanded on irradiated rat feeder cells as described previously (135). The expanded cells were separated from feeder cells and grown in sandwich culture between collagen and matrigel. Media was supplemented with HGF (40ng/ml), EGF (25ng/ml) or Dex (100nM) as previously described (81). The cells were harvested after two weeks. (B**)** RT- PCR analyses of Albumin and β-Actin expression on differentiated cells. Primary hepatocytes and expanded EpCAM+CD49f+ cells prior to differentiation were used as positive and negative controls, respectively. "Control" denotes cells grown between the gels in the absence of any growth factor or steroid. EpCAM+CD49f+ cells grown between collagen and matrigel in the presence of HGF, EGF and Dex express albumin.

5.3.2 Microarray analyses of gallbladder cells and hepacotytes

The specific transcription factors required for the reprogramming of gallbladder cells into hepatocytes are not known. In order to elucidate which of these transcription factors would be necessary for hepatic differentiation, oligonucleotide microarrays were performed on primary

mouse hepatocytes, expanded mouse gallbladder stem cells and primary mouse gallbladder epithelial (EpCAM+) cells. Our rationale here is that as stem cells are more amenable to reprogramming than differentiated cells (102), the expanded gallbladder stem cells would be a better target for reprogramming than total gallbladder epithelial cells. In order to test this hypothesis, we performed microarray analyses on both primary and expanded gallbladder cells.

Unsupervised hierarchical clustering analyses demonstrated that the transcriptomes of expanded gallbladder stem cells is more similar to that of hepatocytes than the transcriptomes of primary gallbladder cells is (Figure 25A). Next we ran unpaired t-tests between (hepatocytes and primary gallbladder cells) and (hepatocytes and expanded gallbladder stem cells) in BRB-ArrayTools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team ($p<0.001$). We found significantly fewer transcripts different between expanded gallbladder stem cells and hepatocytes (3058 out of 34,842 total transcripts) than between primary gallbladder cells and hepatocytes (5513 out of 34,842 total transcripts) (Figure 25B). These data further corroborate the hierarchical clustering results. We then investigated the basis of the similarity between expanded gallbladder stem cells and hepatocytes. Expanded gallbladder stem cells represent a subpopulation of primary gallbladder epithelial cells capable of long-term clonal expansion and differentiation *in vitro* (135). Therefore, their expression profile would be a subset of that of primary epithelial cells. By extension, transcripts that were significantly different between expanded gallbladder stem cells and hepatocytes would be a subset of those that were different between total primary gallbladder cells and hepatocytes. In line with this hypothesis, we found a significant (~70%) number of transcripts present in the (hepatocytes and expanded gallbladder stem cells) dataset were also present (hepatocytes and primary gallbladder cells) dataset (Figure 25C). In all, these data confirm that expanded gallbladder stem cells

would be more amenable to hepatic reprogramming than primary gallbladder cells. Consequently, we focused on the dataset of transcripts significantly different between hepatocytes and expanded gallbladder stem cells.

5.3.3 Candidate transcription factors for hepatic reprogramming

We mined the dataset of transcripts significantly different between hepatocytes and expanded gallbladder stem cells to identify transcription factors. 175 transcription factors were different, of which 139 had a fold change greater than two. 47 out of these 139 transcription factors were more highly expressed in hepatocytes compared to expanded gallbladder stem cells (Figure 25D). As this dataset was relatively large, we focused on the twenty most differentially expressed transcription factors. In this process, we identified three transcription factors - CCAAT-enhancer binding protein alpha (C/EBPα), FoxA3 and HNF4α- that could be important for reprogramming. Differential expression of C/EBPα, FoxA3 and HNF4α was validated by RT-PCR analysis (Figure 25E). Taken together these data suggest that C/EBPα, FoxA3 and HNF4α are interesting candidates for the direct reprogramming of mouse gallbladder stem cells into hepatocytes.

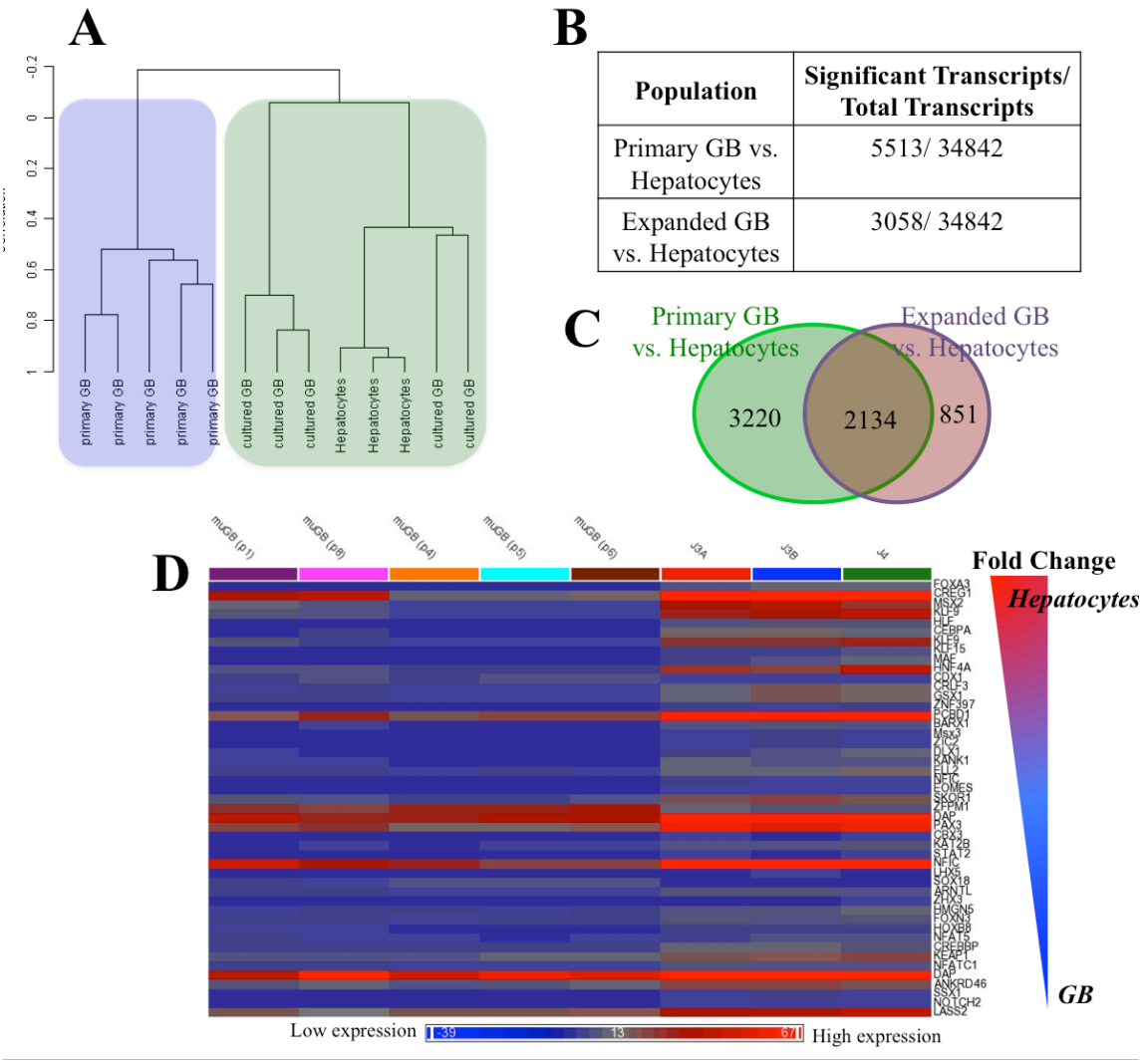


Figure 25: Candidate transcription factors for the direct reprogramming of gallbladder stem cells into hepatocytes

(A) Expanded EpCAM+CD49f+ gallbladder (GB) stem cells have a transcriptome that is more similar to hepatocytes than that of bulk primary GB epithelial (EpCAM+) cells. Dendograms were generated in BRB-ArrayTools ($p<0.001$) by unsupervised clustering of primary GB,

cultured GB and hepatocyte datasets, by centered correlation and average linkage methods. (B) Differentially regulated transcripts between (primary GB vs. hepatocytes) and (cultured GB vs. hepatocytes) were defined by running unpaired t-tests in BRB-ArrayTools ($p<0.001$). 86% of all significant transcripts for each analysis represent Mapped IDs. (C) Significant overlaps between transcripts present in (primary GB vs. hepatocytes) and (cultured GB vs. hepatocytes) datasets. (D) Heatmaps of the transcription factors significantly upregulated ($p<0.001$; fold change ≥ 2) in hepatocytes compared to expanded EpCAM+CD49f+ GB stem cells ranked by fold change. Red indicates expression higher relative expression; blue indicates lower relative expression.

5.4 CONCLUSION & FUTURE DIRECTIONS

There is a tremendous need to develop alternative cell-based therapy to treat end stage liver disease. The use of gallbladder stem cells to treat liver disease has two particular advantages. The adult gallbladder is a non-vital organ, and removal of the gallbladder is a common surgery (80) allowing for a ready source of donor tissue. In addition, the shared ontogeny of the gallbladder and liver suggest that a gallbladder cell would be relatively easily reprogrammed into a hepatocyte than a fibroblast would. For these reasons, we attempted a proof-of-concept experiment to determine if gallbladder cells could differentiate into hepatocye-like cells and determined candidate transcription factors that would allow for direct reprogramming.

Using a previously published culture protocol (81), we found that adult mouse gallbladder stem cells (135), exhibit albumin gene expression *in vitro*. However, these cells do not lose expression of the bile duct marker CK19 after differentiation suggesting that their phenotype is probably closer to that of hepatoblasts rather than hepatocytes. In the future,

albumin-expressing gallbladder stem cells could be transplanted into *Fah*$^{-/-}$ mice to determine if they could complete hepatic differentiation *in vivo* and subsequently repopulate the livers. Furthermore, it is also possible that the final cell population following *in vitro* differentiation is heterogeneous, with only some gallbladder cells expressing albumin. To track and eventually separate the albumin-expressing cells, gallbladder stem cells can be transfected with a construct containing the Albumin promoter/enhancer upstream of a fluorescent reporter (156). The Albumin promoter traces albumin expressing cells such as mature hepatocytes and hepatocyte precursors such as hepatoblasts (156). In this manner, the albumin-expressing cells can be isolated following differentiation *in vitro* and injected into *Fah*$^{-/-}$ mice.

We identified three transcription factors -C/EBPα, FoxA3 and HNF4α- that are more highly expressed in hepatocytes than gallbladder stem cells. These data suggest that these factors could be important for the hepatic reprogramming of gallbladder stem cells. Each of these factors has been shown to be important for hepatocyte function and development (87, 157-159). In addition, HNF4α and FoxA3 have been previously used in the hepatic reprogramming of fibroblasts (84, 85). These data suggest a strong symmetry in the transcription factors required for hepatic specification. Given the shared ontogeny between gallbladder cells and hepatocytes, we hypothesize that a single transcription factor would be sufficient to differentiate gallbladder stem cells into hepatocytes. Finally, these studies would set the stage for the differentiation of human gallbladder stem cells into hepatocytes. If successful, these experiments will have important ramifications for cell-based therapy to treat liver disease.

6.0 DISCUSSION

The identification of bile duct stem cells holds great promise for basic biology and cell based therapy. This study has focused on the identification of gallbladder and IHBD stem cells, in order to compare them to each other as well as for potential cell-based therapy to treat liver disease. We have developed *in vitro* expansion and differentiation assays for the evaluation of adult mouse and fetal human gallbladder stem cells and adult mouse IHBD stem cells. Finally, we use microarray analysis to compare mouse gallbladder and IHBD stem cells to each other, and to identify transcription factors that can be used in the reprogramming of gallbladder stem cells into hepatocytes.

6.1 IDENTIFICATION OF GALLBLADDER STEM CELLS

There is a paucity of data for the identification of stem cells in the uninjured gallbladder. Accordingly, there is a lack of *in vitro* and *in vivo* assays for the study of gallbladder stem cells. We found that irradiated cells of the rat mammary tumor cell line LA7 could be used feeder cells to support expansion of adult mouse and fetal human gallbladder epithelial cells. These cells have previously been used to expand mouse mammary epithelial cells (95), porcine (96) and human bladder epithelial cells (97) and human colon cancer cells (98) and appear to support expansion of ductal epithelial cells.

Stem cells have the capacity of self-renewal and we predicted that expansion *in vitro* would select for cells capable of self-renewal thereby enriching for primitive or stem cells. As there are no markers to separate mature gallbladder cells from immature ones, we used the LDA –a colony forming assay- to characterize expanded gallbladder cells as stem cells. In this manner, we determined that CD49f is a mouse gallbladder stem cell marker and that CD13 and CD44 are fetal human gallbladder stem cell markers. These data corroborate previous results describing CD49f as stem cell marker in the mouse liver (117, 118) and the mammary gland (119, 120). Similarly, CD44 has been implicated as a human gallbladder cancer stem cell marker (46, 47). Mouse EpCAM+CD49f^{hi} cells expanded *in vitro* and exhibited single cell self-renewal and lineage commitment. Similarly human EpCAM+CD13+CD44+ cells were the most enriched for *in vitro* colony forming ability, expanded from singe cells and differentiated into gallbladder-like structures. Important to both studies, we first used EpCAM to identify gallbladder epithelial cells, thereby preventing contamination by mesenchymal, hematopoietic or other non-epithelial cells. This is especially important as we detected EpCAM-CD49f^{hi} cells in primary mouse gallbladder, and EpCAM-CD13+ and EpCAM-CD44+ cells in primary human gallbladder.

Interestingly, we did not identify CD49f as a fetal human gallbladder stem cell marker and CD13 and CD44 as adult mouse gallbladder stem cell markers. CD13 and CD44 were not expressed on primary and expanded mouse gallbladder epithelial cells (Table 2). Similarly, CD49f was not heterogeneous on primary human fetal gallbladder epithelial cells (Table 8). In addition, EpCAM+CD49f^{hi} cells from mouse gallbladders formed flat and glandular colonies *in vitro* at first expansion (p0), and only a fraction of flat colonies could expand past p0 (Figure 6). Therefore, the EpCAM+CD49f^{hi} population in primary mouse gallbladder is itself

heterogeneous with only a subpopulation of cells capable of self-renewal past p0. Either way, we did not notice a similar morphological heterogeneity with human fetal gallbladder cells. Last, the morphogenesis assays of mouse and human gallbladder stem cells were different. Unlike the mouse gallbladder stem cells, we did not observe cyst formation when human fetal gallbladder cells were grown in matrigel. These differences in data might be because we are comparing adult mouse gallbladder and fetal human gallbladder. However, it is also possible that the stem cell populations in mouse and human gallbladders are different from each other. It is noteworthy that rats do not have a gallbladder (6) making additional comparisons between rodent and human gallbladders difficult.

6.1.1 Gallbladder stem cells as committed progenitor cells

Stem cells are considered to be capable of asymmetric cell division (160). While this has not been shown for liver or gallbladder stem cells, our hypothesis was that gallbladder stem cells would be able to reconstitute the heterogeneity of the primary gallbladder epithelium. Specifically, we expected expanded EpCAM+CD49f+ mouse gallbladder stem cells to form EpCAM+CD49f- or EpCAM+CD49f^{lo} cells after differentiation *in vitro* or *in vivo*. However, analysis of cysts in matrigel culture and after engraftment *in vivo* (Figure 9) show that EpCAM+CD49f+ cells remained EpCAM+CD49f+. We are currently evaluating if EpCAM+CD44+CD13+ cells can engraft *in vivo* in the subcutaneous space of mice (123) similar to mouse gallbladder stem cells, and will also evaluate expression of CD13 and CD44 on the human cysts *in vitro*.

In all, these data suggest that gallbladder stem cells are committed progenitor cells that might not be able to fully reconstitute the heterogeneity of gallbladder epithelium, as has been

suggested before (50). However, more plausibly we have not yet identified a definitive gallbladder differentiation assay.

6.2 IDENTIFICATION OF IHBD STEM CELLS

The identification of resident stem cells in the uninjured liver has been controversial. Using the expansion and differentiation assays we developed for the study of mouse gallbladder stem cells (135), we were able to successfully expand mouse IHBD epithelial cells *in vitro* and demonstrate that a subpopulation of these cells exhibits single cell self-renewal and lineage commitment. Mouse and human IHBD cells have been expanded in monolayer culture on collagen gels (53, 161-163). However, expansion of IHBD epithelial cells, especially in the mouse is hindered by the fact that these cells are very rare in primary liver (8). Similarly, we found that IHBD epithelial cells in adult liver were rare (Figure 11A). Using a combination of MACS for EpCAM and expansion on LA7 feeder cells we observed a robust expansion of IHBD epithelial cells. Similar expansion of epithelial cells have been noted with IHBD explant culture (164), wherein the ducts themselves are dissected out following perfusion and grown on collagen gels. However, for the obvious reasons these techniques are not amenable to the evaluation of stem cell markers in IHBD epithelial cells.

We were not able to identify specific markers that highlighted heterogeneity in the IHBD epithelial cells. In the future, microarray analysis could be used in order to identify specific cell surface markers that enrich for the specific subpopulations of EpCAM+ IHBD cells capable of colony formation similar to a previous study with fetal mouse liver stem cells (141). Last, specific antibodies could be generated against EpCAM+ IHBD cells, similar to a study by

Dorrell et al. (74), which might allow for the identification of heterogeneous subpopulations within EpCAM+ IHBD cells.

6.2.1 IHBD stem cells as committed progenitor cells

We found that the expanded EpCAM+ IHBD stem cells were unipotent and were not able to generate hepatocytes *in vivo*. These data suggest that pure (>98%) EpCAM+ IHBD epithelial cells are not capable of *de novo* hepatocyte differentiation. Transplantation experiments are being repeated with primary EpCAM+ IHBD cells. These experiments are especially important as two previous studies report the engraftment of primary liver stem cells in *Fah*$^{-/-}$ mice (70, 72). One possible explanation for the discrepancy in the results might be the purity of the donor cells injected. The two foregoing studies do not preclude the possibility of hepatocyte contamination in the donor cell preparation. Given that IHBD cells are a rare population in the small cell fraction from primary liver isolate, it would be necessary to obtain a pure (>98%) primary EpCAM+ IHBD cell preparation prior to injection. Using a combination of MACS for EpCAM following by cell sorting, we are currently optimizing a protocol for the isolation of pure (>98%) primary EpCAM+ IHBD cells. In addition, prior to transplant these cells will be visualized on a microscope to confirm the absence of any large cells (hepatocytes).

We also found that not all IHBD cells isolated by liver perfusion (CK19+ cells in the small cell fraction) were EpCAM+ (Table 18); most (85-90%) but not all of the CK19+ cells are EpCAM+. These data raise the intriguing possibility that there is an EpCAM- IHBD cell fraction that is bipotent and capable of *de novo* hepatocyte differentiation. EpCAM- IHBD cells from small cell fractions were sorted on LA7 feeder cells but did not exhibit *in vitro* expansion.

Apart from differentiation into hepatocytes, we did not note differentiation of small cuboidal IHBD cells into larger columnar IHBD cells (Figure 16). The perfusion protocol that we employ (109), followed by subsequent isolation of the small cell fraction, results in the isolation of small bile duct cells (53) (Figure 16C). Small cuboidal IHBD cells around the Canals of Hering have a different morphology, phenotypic profile (52) and global gene expression profile (51) than larger columnar IHBD cells around the larger bile ducts. Similar to the identification of mouse gallbladder stem cells, these data indicate that IHBD stem cells are committed progenitor cells. However, it is more plausible again that we as yet have not identified a definitive IHBD differentiation assay.

6.3 DIFFERENCES BETWEEN GALLBLADDER STEM CELLS AND IHBD STEM CELLS

It has recently been shown that the IHBD and EHBD system (including the gallbladder) develop from separate progenitor cells (20). We found that expression of CD49f, CD49e, CD81, CD26, CD54 and CD166 was different between bulk primary IHBD and gallbladder cells suggesting that the IHBD and EHBD cells *in vivo* are different (Figure 10). In addition, microarray analyses indicated that the transcriptomes of expanded EpCAM+CD49f+ cells and expanded EpCAM+ IHBD cells were different. The goal of microarray was to determine if the gallbladder stem cells were different than the IHBD cells. Expanded EpCAM+CD49f+ gallbladder cells (>passage 0) represent a purer stem cell population than primary EpCAM+CD49f^{hi} cells. The latter forms both flat and glandular colonies and only a fraction of the flat can self-renew as described above. Therefore, we used the expanded EpCAM+CD49f+

cells at later passage in the microarray. However, neither cell population is a pure stem cell population as not all of the constituent cells can expand from single cells.

The major groups of differentially expressed genes were cytochrome P450 genes, glutathione-S-transferase and the solute carrier family genes. Also interferon (IFN)-inducible protein 27 was differentially expressed between gallbladder and IHBD cells. Interestingly, expression of CD54 is known to be immunologically mediated (129). The immunologic properties of bile duct cells have long been considered. They are the primary site of damage in inflammatory diseases such as primary biliary cirrhosis (130) and biliary atresia (21) and in liver allograft rejection (131). The differential expression of an IFN-inducible protein and CD54 indicates that the potential immunologic properties of IHBD and gallbladder cells could be different. These data are especially important as it is becoming clearer that that bile and bile duct cells have active immunological roles in innate and adaptive immunity (reviewed in (8)).

Interestingly, we found phenotypic differences in expanded human fetal gallbladder stem cells and IHBD cells (Figure 23C) suggesting that these two cell types are different as well. It is noteworthy that we did not observe phenotypic differences between expanded mouse EpCAM+CD49f+ cells and expanded mouse EpCAM+ IHBD cells (Table 2). We are currently evaluating the global gene expression profile of expanded human fetal gallbladder stem cells and IHBD cells by oligonucleotide microarray. Similar to the expanded mouse EpCAM+ IHBD cells, it is possible that expanded human fetal IHBD cells might satisfy the stem cell properties of single cell self-renewal and lineage commitment. However, these experiments are beyond the scope of the current study.

6.4 GALLBLADDER STEM CELLS AS POTENTIAL CELL-BASED THERAPY

In this study we have identified a resident stem cell population in adult mouse gallbladder and fetal human gallbladder. These cells could be important for cell-based therapy for liver disease as discussed earlier, for two seasons: their ontogeny and availability. The gallbladder and liver are derived from the central foregut endoderm (12) suggesting that a gallbladder stem cells would be more easily reprogrammed into a hepatocyte than a fibroblast could. In addition, the gallbladder is a small non-vital organ and removal of the gallbladder is routine (80). This availability of tissue along with the putative plasticity of gallbladder stem cells into hepatocytes makes these cells attractive targets for cell-based therapy for liver disease.

We found that expanded EpCAM+CD49f+ gallbladder stem cells were capable of albumin secretion (Figure 24) in an *in vitro* assay for hepatocyte differentiation. However, these cells continued to express CK19 suggesting that they were not mature hepatocytes. In addition, recent data describing the reprogramming of fibroblasts into hepatocytes (84, 85), suggest that directed differentiation of somatic cells with transcription factors might yield mature hepatocytes.

We performed oligonucleotide microarrays on primary EpCAM+ gallbladder epithelial cells, expanded EpCAM+CD49f+ cells and primary hepatocytes to identify candidate transcription factors for the reprogramming of gallbladder stem cells into hepatocytes. First we found that the transcriptomes of expanded gallbladder stem cells is more similar to that of hepatocytes than the transcriptomes of primary gallbladder cells is (Figure 25A & B). These data corroborate the notion that stem cells are more amenable to reprogramming than differentiated cells are (102). Accordingly, we focused on the transcription factors that were significantly different between expanded gallbladder stem cells and hepatocytes.

We identified three transcription factors -C/EBPα, FoxA3 and HNF4α- that are more highly expressed in hepatocytes than gallbladder stem cells. These data suggest that these factors could be important for the hepatic reprogramming of gallbladder stem cells. Each of these factors has been shown to be important for hepatocyte function and development (87, 157-159). In addition, HNF4α and FoxA3 have been previously used in the hepatic reprogramming of fibroblasts (84, 85). These data suggest a strong symmetry in the transcription factors required for hepatic specification. Given the shared ontogeny between gallbladder cells and hepatocytes, we hypothesize that a single transcription factor would be sufficient to differentiate gallbladder stem cells into hepatocytes. These data would suggest that gallbladder stem cells are more easily reprogrammed into hepatocytes than fibroblasts are. Finally, the reprogramming of mouse gallbladder stem cells into hepatocytes would set the stage for reprogramming studies with human gallbladder cells. Initially these experiments can be performed with expanded human fetal gallbladder stem cells but can be continued with adult human gallbladder stem cells.

Last, gallbladder stem cells can be used to treat biliary atresia as has been noted with hepatic progenitor cells (132) and also differentiated into endocrine cells. The latter could be important, as it is now known that the EHBD system and ventral pancreas develop from a share progenitor (20). Sumazaki et al. (13) and Fukuda et al. (14) have observed ectopic endocrine cells in the EHBD of *Hes1*$^{-/-}$ deficient mice and knockout mice, respectively. These data support the notion that gallbladder stem cells have plasticity towards endocrine cells.

6.5 FUTURE DIRECTIONS

The expansion and differentiation assays described above could be used to better understand the biology of resident stem cell populations in the gallbladder and liver. LA7 feeder cells provide a robust and reproducible means to expand gallbladder epithelial cells *in vitro*. Furthermore, we have also shown that mouse gallbladder and IHBD cells do not fuse with the feeder cells (Figure 4 & 14). Similar analyses have been performed with human fetal gallbladder cells. However, expansion of stem cells on a xenogeneic cell line could be a potential limitation in downstream applications, especially candidate cell-based therapies. Similar to IHBD cells, collagen gels have typically been used for the monolayer cell culture of gallbladder cells (reviewed in (103)). These methods have been used for expansion of canine (165), rabbit (127, 128, 151) and human (94, 129, 146) gallbladder cells. In the future, conditioned medium from LA7 cells could be used to potentially stimulate expansion of human and mouse gallbladder and IHBD cells on collagen gels. In order to select for epithelial cell expansion, MACS enrichment of EpCAM+ cells could be used to enrich for epithelial cells directly from primary gallbladder. As IHBD cells are rare in the adult mouse liver, multiple MACS columns might be necessary to obtain a pure population of primary EpCAM+ IHBD cells. In addition, LA7 cells are known to secrete TGFα and bFGF (166), and the effects of these growth factors can be evaluated directly on gallbladder and IHBD cells growing on collagen gels.

Cyst formation by mouse gallbladder and IHBD cells and human gallbladder cells can also be used to further investigate differentiation of the gallbladder cells. In the future, primary and expanded gallbladder cells and the cysts could be screened for phenotypic markers of *in vivo* function. Candidates are Aquaporins (AQP) (water transport) such as AQP1 and AQP8

(167), sterol transporters (cholesterol transport) –ABCG5 and ABCG8 (168, 169)— and mucins (170). In this manner, various markers of gallbladder function can be linked to cells with demonstrable stem cell properties allowing for a complete characterization of the gallbladder. Finally, cyst formation can be modulated by various growth factors and cytokines similar to a previous study with a bipotent hepatoblast cell line (107).

6.6 CONCLUSIONS

In this study we focus on the identification of resident stem cells in the gallbladder and liver. First we developed *in vitro* expansion and differentiation to evaluate mouse gallbladder stem cells and then adapted them to study adult mouse IHBD and human fetal gallbladder stem cells. We have demonstrated that adult mouse gallbladder stem cells are distinct or unique compare to IHBD stem cells, and have obtained preliminary data for the reprogramming of the mouse gallbladder stem cells into hepatocytes.

BIBLIOGRAPHY

1. Strazzabosco M, Fabris L. Functional anatomy of normal bile ducts. Anat Rec (Hoboken)2008 Jun;291(6):653-60.
2. Hand BH. Anatomy and function of the extrahepatic biliary system. Clin Gastroenterol1973 Jan;2(1):3-29.
3. Cardinale V, Wang Y, Carpino G, Cui CB, Gatto M, Rossi M, Berloco PB, Cantafora A, Wauthier E, Furth ME, Inverardi L, Dominguez-Bendala J, Ricordi C, Gerber D, Gaudio E, Alvaro D, Reid L. Multipotent stem/progenitor cells in human biliary tree give rise to hepatocytes, cholangiocytes, and pancreatic islets. Hepatology2011 Dec;54(6):2159-72.
4. Carey MC, Cahalane MJ. Whither biliary sludge? Gastroenterology1988 Aug;95(2):508-23.
5. Frizzell RA, Heintze K. Transport functions of the gallbladder. Int Rev Physiol1980;21:221-47.
6. McMaster PD. DO SPECIES LACKING A GALL BLADDER POSSESS ITS FUNCTIONAL EQUIVALENT? J Exp Med1922 Jan 31;35(2):127-40.
7. Fitz JG. Regulation of cholangiocyte secretion. Semin Liver Dis2002 Aug;22(3):241-9.
8. Reynoso-Paz S, Coppel RL, Mackay IR, Bass NM, Ansari AA, Gershwin ME. The immunobiology of bile and biliary epithelium. Hepatology1999 Aug;30(2):351-7.
9. Shiojiri N. Development and differentiation of bile ducts in the mammalian liver. Microsc Res Tech1997 Nov 15;39(4):328-35.
10. Zaret KS, Grompe M. Generation and regeneration of cells of the liver and pancreas. Science2008 Dec 5;322(5907):1490-4.
11. Tremblay KD, Zaret KS. Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. Dev Biol2005 Apr 1;280(1):87-99.
12. Roskams T, Desmet V. Embryology of extra- and intrahepatic bile ducts, the ductal plate. Anat Rec (Hoboken)2008 Jun;291(6):628-35.
13. Sumazaki R, Shiojiri N, Isoyama S, Masu M, Keino-Masu K, Osawa M, Nakauchi H, Kageyama R, Matsui A. Conversion of biliary system to pancreatic tissue in Hes1-deficient mice. Nat Genet2004 Jan;36(1):83-7.
14. Fukuda A, Kawaguchi Y, Furuyama K, Kodama S, Horiguchi M, Kuhara T, Koizumi M, Boyer DF, Fujimoto K, Doi R, Kageyama R, Wright CV, Chiba T. Ectopic pancreas formation in Hes1 -knockout mice reveals plasticity of endodermal progenitors of the gut, bile duct, and pancreas. J Clin Invest2006 Jun;116(6):1484-93.
15. Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T. The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology2004 Dec;127(6):1775-86.

16. Raynaud P, Carpentier R, Antoniou A, Lemaigre FP. Biliary differentiation and bile duct morphogenesis in development and disease. Int J Biochem Cell Biol2011 Feb;43(2):245-56.
17. Ashraf A, Abdullatif H, Hardin W, Moates JM. Unusual case of neonatal diabetes mellitus due to congenital pancreas agenesis. Pediatr Diabetes2005 Dec;6(4):239-43.
18. Heij HA, Niessen GJ. Annular pancreas associated with congenital absence of the gallbladder. J Pediatr Surg1987 Nov;22(11):1033.
19. Mehes K, Vamos K, Goda M. Agenesis of pancreas and gall-bladder in an infant of incest. Acta Paediatr Acad Sci Hung1976;17(3):175-6.
20. Spence JR, Lange AW, Lin SC, Kaestner KH, Lowy AM, Kim I, Whitsett JA, Wells JM. Sox17 regulates organ lineage segregation of ventral foregut progenitor cells. Dev Cell2009 Jul;17(1):62-74.
21. Bassett MD, Murray KF. Biliary atresia: recent progress. J Clin Gastroenterol2008 Jul;42(6):720-9.
22. Mieli-Vergani G, Vergani D. Biliary atresia. Semin Immunopathol2009 Sep;31(3):371-81.
23. Zong Y, Stanger BZ. Molecular mechanisms of bile duct development. Int J Biochem Cell Biol2011 Jul 1.
24. McDiarmid SV, Anand R, Lindblad AS. Studies of Pediatric Liver Transplantation: 2002 update. An overview of demographics, indications, timing, and immunosuppressive practices in pediatric liver transplantation in the United States and Canada. Pediatr Transplant2004 Jun;8(3):284-94.
25. Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, Qi M, Trask BJ, Kuo WL, Cochran J, Costa T, Pierpont ME, Rand EB, Piccoli DA, Hood L, Spinner NB. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat Genet1997 Jul;16(3):243-51.
26. Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, Piccoli DA, Meltzer PS, Spinner NB, Collins FS, Chandrasekharappa SC. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet1997 Jul;16(3):235-42.
27. Lee SP, Scott AJ. The evolution of morphologic changes in the gallbladder before stone formation in mice fed a cholesterol-cholic acid diet. Am J Pathol1982 Jul;108(1):1-8.
28. Albores-Saavedra J, Galliani C, Chable-Montero F, Batich K, Henson DE. Mucin-containing Rokitansky-Aschoff sinuses with extracellular mucin deposits simulating mucinous carcinoma of the gallbladder. Am J Surg Pathol2009 Nov;33(11):1633-8.
29. Albores-Saavedra J, Shukla D, Carrick K, Henson DE. In situ and invasive adenocarcinomas of the gallbladder extending into or arising from Rokitansky-Aschoff sinuses: a clinicopathologic study of 49 cases. Am J Surg Pathol2004 May;28(5):621-8.
30. Kim JH, Jeong IH, Han JH, Hwang JC, Yoo BM, Kim MW, Kim WH. Clinical/pathological analysis of gallbladder adenomyomatosis; type and pathogenesis. Hepatogastroenterology2010 May-Jun;57(99-100):420-5.
31. Baig SJ, Biswas S, Das S, Basu K, Chattopadhyay G. Histopathological changes in gallbladder mucosa in cholelithiasis: correlation with chemical composition of gallstones. Trop Gastroenterol2002 Jan-Mar;23(1):25-7.
32. Cariati A, Cetta F. Rokitansky-Aschoff sinuses of the gallbladder are associated with black pigment gallstone formation: a scanning electron microscopy study. Ultrastruct Pathol2003 Jul-Aug;27(4):265-70.

33. Luciano L, Reale E. The human gallbladder. In: Riva A, Motta PM, editors. Ultrastructure of the Extraparietal Glands of the Digetive Tract: Kluwer Academic Publishers; 1990. p. 231-48.
34. Luciano L, Reale E. A new cell type ("brush cell") in the gall bladder epithelium of the mouse. J Submicrosc Cytol1969;1:43-52.
35. Gilloteaux J, Pomerants B, Kelly TR. Human gallbladder mucosa ultrastructure: evidence of intraepithelial nerve structures. Am J Anat1989 Apr;184(4):321-33.
36. Luciano L, Reale E. Presence of brush cells in the mouse gallbladder. Microsc Res Tech1997 Sep 15;38(6):598-608.
37. Lamote J, Willems G. DNA synthesis, cell proliferation index in normal and abnormal gallbladder epithelium. Microsc Res Tech1997 Sep 15;38(6):609-15.
38. Irie T, Asahina K, Shimizu-Saito K, Teramoto K, Arii S, Teraoka H. Hepatic progenitor cells in the mouse extrahepatic bile duct after a bile duct ligation. Stem Cells Dev2007 Dec;16(6):979-87.
39. Carpino G, Cardinale V, Onori P, Franchitto A, Berloco PB, Rossi M, Wang Y, Semeraro R, Anceschi M, Brunelli R, Alvaro D, Reid LM, Gaudio E. Biliary tree stem/progenitor cells in glands of extrahepatic and intraheptic bile ducts: an anatomical in situ study yielding evidence of maturational lineages. J Anat2012 Feb;220(2):186-99.
40. Terada T, Nakanuma Y. Development of human intrahepatic peribiliary glands. Histological, keratin immunohistochemical, and mucus histochemical analyses. Lab Invest1993 Mar;68(3):261-9.
41. Nakanuma Y, Katayanagi K, Terada T, Saito K. Intrahepatic peribiliary glands of humans. I. Anatomy, development and presumed functions. J Gastroenterol Hepatol1994 Jan-Feb;9(1):75-9.
42. Terada T, Kida T, Nakanuma Y. Extrahepatic peribiliary glands express alpha-amylase isozymes, trypsin and pancreatic lipase: an immunohistochemical analysis. Hepatology1993 Oct;18(4):803-8.
43. Miller G, Jarnagin WR. Gallbladder carcinoma. Eur J Surg Oncol2008 Mar;34(3):306-12.
44. Levy AD, Murakata LA, Rohrmann CA, Jr. Gallbladder carcinoma: radiologic-pathologic correlation. Radiographics2001 Mar-Apr;21(2):295-314; questionnaire, 549-55.
45. Roa I, de Aretxabala X, Araya JC, Villaseca M, Roa J, Guzman P. [Incipient gallbladder carcinoma. Clinical and pathological study and prognosis in 196 cases]. Rev Med Chil2001 Oct;129(10):1113-20.
46. Shi C, Tian R, Wang M, Wang X, Jiang J, Zhang Z, Li X, He Z, Gong W, Qin R. CD44+ CD133+ population exhibits cancer stem cell-like characteristics in human gallbladder carcinoma. Cancer Biol Ther2010 Dec 1;10(11):1182-90.
47. Shi CJ, Gao J, Wang M, Wang X, Tian R, Zhu F, Shen M, Qin RY. CD133(+) gallbladder carcinoma cells exhibit self-renewal ability and tumorigenicity. World J Gastroenterol2011 Jun 28;17(24):2965-71.
48. Roskams TA, Theise ND, Balabaud C, Bhagat G, Bhathal PS, Bioulac-Sage P, Brunt EM, Crawford JM, Crosby HA, Desmet V, Finegold MJ, Geller SA, Gouw AS, Hytiroglou P, Knisely AS, Kojiro M, Lefkowitch JH, Nakanuma Y, Olynyk JK, Park YN, Portmann B, Saxena R, Scheuer PJ, Strain AJ, Thung SN, Wanless IR, West AB. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology2004 Jun;39(6):1739-45.

49. Glaser S, Francis H, Demorrow S, Lesage G, Fava G, Marzioni M, Venter J, Alpini G. Heterogeneity of the intrahepatic biliary epithelium. World J Gastroenterol2006 Jun 14;12(22):3523-36.
50. Cardinale V, Wang Y, Carpino G, Mendel G, Alpini G, Gaudio E, Reid LM, Alvaro D. The biliary tree-a reservoir of multipotent stem cells. Nat Rev Gastroenterol Hepatol2012 Feb 28.
51. Ueno Y, Alpini G, Yahagi K, Kanno N, Moritoki Y, Fukushima K, Glaser S, LeSage G, Shimosegawa T. Evaluation of differential gene expression by microarray analysis in small and large cholangiocytes isolated from normal mice. Liver Int2003 Dec;23(6):449-59.
52. Glaser SS, Gaudio E, Rao A, Pierce LM, Onori P, Franchitto A, Francis HL, Dostal DE, Venter JK, DeMorrow S, Mancinelli R, Carpino G, Alvaro D, Kopriva SE, Savage JM, Alpini GD. Morphological and functional heterogeneity of the mouse intrahepatic biliary epithelium. Lab Invest2009 Apr;89(4):456-69.
53. Gall JA, Bhathal PS. Isolation and culture of intrahepatic bile ducts and its application in assessing putative inducers of biliary epithelial cell hyperplasia. Br J Exp Pathol1987 Aug;68(4):501-10.
54. Gall JAM, Bhathal PS. A morphological and stereological analysis of the rat liver following ligation of the common bile duct. Pathology1984;16:481-2.
55. Riehle KJ, Dan YY, Campbell JS, Fausto N. New concepts in liver regeneration. J Gastroenterol Hepatol2011 Jan;26 Suppl 1(2011):203-12.
56. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology2006 Feb;43(2 Suppl 1):S45-53.
57. Duncan AW, Dorrell C, Grompe M. Stem Cells and Liver Regeneration. Gastroenterology2009 May 24.
58. Michalopoulos GK. Liver regeneration. J Cell Physiol2007 Nov;213(2):286-300.
59. Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T, Hosokawa S, Elbahrawy A, Soeda T, Koizumi M, Masui T, Kawaguchi M, Takaori K, Doi R, Nishi E, Kakinoki R, Deng JM, Behringer RR, Nakamura T, Uemoto S. Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. Nat Genet2010 Nov 28.
60. Malato Y, Naqvi S, Schurmann N, Ng R, Wang B, Zape J, Kay MA, Grimm D, Willenbring H. Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. J Clin Invest2011 Dec;121(12):4850-60.
61. Wilson JW, Leduc EH. Role of cholangioles in restoration of the liver of the mouse after dietary injury. J Pathol Bacteriol1958 Oct;76(2):441-9.
62. Kuwahara R, Kofman AV, Landis CS, Swenson ES, Barendswaard E, Theise ND. The hepatic stem cell niche: identification by label-retaining cell assay. Hepatology2008 Jun;47(6):1994-2002.
63. Schmelzer E, Zhang L, Bruce A, Wauthier E, Ludlow J, Yao HL, Moss N, Melhem A, McClelland R, Turner W, Kulik M, Sherwood S, Tallheden T, Cheng N, Furth ME, Reid LM. Human hepatic stem cells from fetal and postnatal donors. J Exp Med2007 Aug 6;204(8):1973-87.
64. Okabe M, Tsukahara Y, Tanaka M, Suzuki K, Saito S, Kamiya Y, Tsujimura T, Nakamura K, Miyajima A. Potential hepatic stem cells reside in EpCAM+ cells of normal and injured mouse liver. Development2009 Jun;136(11):1951-60.

65. Duret C, Gerbal-Chaloin S, Ramos J, Fabre JM, Jacquet E, Navarro F, Blanc P, Sa-Cunha A, Maurel P, Daujat-Chavanieu M. Isolation, characterization, and differentiation to hepatocyte-like cells of nonparenchymal epithelial cells from adult human liver. Stem Cells2007 Jul;25(7):1779-90.
66. Schmelzer E, Wauthier E, Reid LM. The phenotypes of pluripotent human hepatic progenitors. Stem Cells2006 Aug;24(8):1852-8.
67. Herrera MB, Bruno S, Buttiglieri S, Tetta C, Gatti S, Deregibus MC, Bussolati B, Camussi G. Isolation and characterization of a stem cell population from adult human liver. Stem Cells2006 Dec;24(12):2840-50.
68. Malhi H, Irani AN, Gagandeep S, Gupta S. Isolation of human progenitor liver epithelial cells with extensive replication capacity and differentiation into mature hepatocytes. J Cell Sci2002 Jul 1;115(Pt 13):2679-88.
69. Suzuki A, Sekiya S, Onishi M, Oshima N, Kiyonari H, Nakauchi H, Taniguchi H. Flow cytometric isolation and clonal identification of self-renewing bipotent hepatic progenitor cells in adult mouse liver. Hepatology2008 Oct 3.
70. Qiu Q, Hernandez JC, Dean AM, Rao PH, Darlington GJ. CD24-Positive Cells from Normal Adult Mouse Liver Are Hepatocyte Progenitor Cells. Stem Cells Dev2011 Oct 21.
71. Shin S, Walton G, Aoki R, Brondell K, Schug J, Fox A, Smirnova O, Dorrell C, Erker L, Chu AS, Wells RG, Grompe M, Greenbaum LE, Kaestner KH. Foxl1-Cre-marked adult hepatic progenitors have clonogenic and bilineage differentiation potential. Genes Dev2011 Jun 1;25(11):1185-92.
72. Dorrell C, Erker L, Schug J, Kopp JL, Canaday PS, Fox AJ, Smirnova O, Duncan AW, Finegold MJ, Sander M, Kaestner KH, Grompe M. Prospective isolation of a bipotential clonogenic liver progenitor cell in adult mice. Genes Dev2011 Jun 1;25(11):1193-203.
73. Schmelzer E, Reid LM. Human telomerase activity, telomerase and telomeric template expression in hepatic stem cells and in livers from fetal and postnatal donors. Eur J Gastroenterol Hepatol2009 Oct;21(10):1191-8.
74. Dorrell C, Erker L, Lanxon-Cookson KM, Abraham SL, Victoroff T, Ro S, Canaday PS, Streeter PR, Grompe M. Surface markers for the murine oval cell response. Hepatology2008 Oct;48(4):1282-91.
75. Kung HC, Hoyert DL, Xu J, Murphy SL. Deaths: final data for 2005. Natl Vital Stat Rep2008 Apr 24;56(10):1-120.
76. Alter HJ, Seeff LB. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. Semin Liver Dis2000;20(1):17-35.
77. Brown RS. Hepatitis C and liver transplantation. Nature2005 Aug 18;436(7053):973-8.
78. UNOS. United Network of Organ Sharing Online [database on the Internet]. [cited April 7th 2009]. Available from: http://www.unos.org/data
.
79. Strom SC, Chowdhury JR, Fox IJ. Hepatocyte transplantation for the treatment of human disease. Semin Liver Dis1999;19(1):39-48.
80. MacFadyen BV, Jr., Vecchio R, Ricardo AE, Mathis CR. Bile duct injury after laparoscopic cholecystectomy. The United States experience. Surg Endosc1998 Apr;12(4):315-21.
81. Kuver R, Savard CE, Lee SK, Haigh WG, Lee SP. Murine gallbladder epithelial cells can differentiate into hepatocyte-like cells in vitro. Am J Physiol Gastrointest Liver Physiol2007 Nov;293(5):G944-55.

82. Kuver R, Savard C, Nguyen TD, Osborne WR, Lee SP. Isolation and long-term culture of gallbladder epithelial cells from wild-type and CF mice. In Vitro Cell Dev Biol Anim1997 Feb;33(2):104-9.
83. Lee SP, Savard CE, Kuver R. Gallbladder epithelial cells that engraft in mouse liver can differentiate into hepatocyte-like cells. Am J Pathol2009 Mar;174(3):842-53.
84. Huang P, He Z, Ji S, Sun H, Xiang D, Liu C, Hu Y, Wang X, Hui L. Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature2011 May 11.
85. Sekiya S, Suzuki A. Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature2011 Jul 21;475(7356):390-3.
86. Willenbring H. A simple code for installing hepatocyte function. Cell Stem Cell2011 Aug 5;9(2):89-91.
87. Kyrmizi I, Hatzis P, Katrakili N, Tronche F, Gonzalez FJ, Talianidis I. Plasticity and expanding complexity of the hepatic transcription factor network during liver development. Genes Dev2006 Aug 15;20(16):2293-305.
88. Grompe M, al-Dhalimy M, Finegold M, Ou CN, Burlingame T, Kennaway NG, Soriano P. Loss of fumarylacetoacetate hydrolase is responsible for the neonatal hepatic dysfunction phenotype of lethal albino mice. Genes Dev1993 Dec;7(12A):2298-307.
89. Grompe M, Lindstedt S, al-Dhalimy M, Kennaway NG, Papaconstantinou J, Torres-Ramos CA, Ou CN, Finegold M. Pharmacological correction of neonatal lethal hepatic dysfunction in a murine model of hereditary tyrosinaemia type I. Nat Genet1995 Aug;10(4):453-60.
90. Lagasse E. Liver Stem Cells. In: Lanza R, Langer R, Vacanti J, editors. Principles of Tissue Engineering. 3rd ed2007. p. 693-704.
91. Wagers AJ, Weissman IL. Plasticity of adult stem cells. Cell2004 Mar 5;116(5):639-48.
92. Wang Y, Yao HL, Cui CB, Wauthier E, Barbier C, Costello MJ, Moss N, Yamauchi M, Sricholpech M, Gerber D, Loboa EG, Reid LM. Paracrine signals from mesenchymal cell populations govern the expansion and differentiation of human hepatic stem cells to adult liver fates. Hepatology2010 Oct;52(4):1443-54.
93. Kubota H, Reid LM. Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. Proc Natl Acad Sci U S A2000 Oct 24;97(22):12132-7.
94. Kobayashi K, Kan M, Yamane I, Ishii M, Toyota T. Primary culture of human gallbladder epithelial cells. Gastroenterol Jpn1991 Jun;26(3):363-9.
95. Ehmann UK, Peterson WD, Jr., Misfeldt DS. To grow mouse mammary epithelial cells in culture. J Cell Biol1984 Mar;98(3):1026-32.
96. Ehmann UK, Terris MK. Long-term culture of porcine bladder epithelial cells. In Vitro Cell Dev Biol Anim2002 Mar;38(3):137-41.
97. Ehmann UK, Terris MK. Juxtacrine stimulation of normal and malignant human bladder epithelial cell proliferation. J Urol2002 Feb;167(2 Pt 1):735-41.
98. Odoux C, Fohrer H, Hoppo T, Guzik L, Stolz DB, Lewis DW, Gollin SM, Gamblin TC, Geller DA, Lagasse E. A stochastic model for cancer stem cell origin in metastatic colon cancer. Cancer Res2008 Sep 1;68(17):6932-41.
99. Fazekas de St G. The evaluation of limiting dilution assays. J Immunol Methods1982 Mar 12;49(2):R11-23.
100. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, Peault B. Isolation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci U S A1992 Apr 1;89(7):2804-8.

101. Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH, Weissman IL. Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci U S A2000 Dec 19;97(26):14720-5.
102. Manohar R, Lagasse E. Transdetermination: a new trend in cellular reprogramming. Mol Ther2009 Jun;17(6):936-8.
103. Nakanuma Y, Katayanagi K, Kawamura Y, Yoshida K. Monolayer and three-dimensional cell culture and living tissue culture of gallbladder epithelium. Microsc Res Tech1997 Oct 1;39(1):71-84.
104. Si-Tayeb K, Lemaigre FP, Duncan SA. Organogenesis and development of the liver. Dev Cell2010 Feb 16;18(2):175-89.
105. Wersto RP, Chrest FJ, Leary JF, Morris C, Stetler-Stevenson MA, Gabrielson E. Doublet discrimination in DNA cell-cycle analysis. Cytometry2001 Oct 15;46(5):296-306.
106. Rao MS, Dwivedi RS, Subbarao V, Usman MI, Scarpelli DG, Nemali MR, Yeldandi A, Thangada S, Kumar S, Reddy JK. Almost total conversion of pancreas to liver in the adult rat: a reliable model to study transdifferentiation. Biochem Biophys Res Commun1988 Oct 14;156(1):131-6.
107. Tanimizu N, Miyajima A, Mostov KE. Liver progenitor cells develop cholangiocyte-type epithelial polarity in three-dimensional culture. Mol Biol Cell2007 Apr;18(4):1472-9.
108. Stolz DB, Ross MA, Salem HM, Mars WM, Michalopoulos GK, Enomoto K. Cationic colloidal silica membrane perturbation as a means of examining changes at the sinusoidal surface during liver regeneration. Am J Pathol1999 Nov;155(5):1487-98.
109. Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol1976;13:29-83.
110. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics2007 Jan;8(1):118-27.
111. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A2001 Apr 24;98(9):5116-21.
112. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A1998 Dec 8;95(25):14863-8.
113. Trzpis M, McLaughlin PM, de Leij LM, Harmsen MC. Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. Am J Pathol2007 Aug;171(2):386-95.
114. de Boer CJ, van Krieken JH, Janssen-van Rhijn CM, Litvinov SV. Expression of Ep-CAM in normal, regenerating, metaplastic, and neoplastic liver. J Pathol1999 Jun;188(2):201-6.
115. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell1982 Nov;31(1):11-24.
116. Zheng YW, Taniguchi H. Diversity of hepatic stem cells in the fetal and adult liver. Semin Liver Dis2003 Nov;23(4):337-48.
117. Tsuchiya A, Heike T, Baba S, Fujino H, Umeda K, Matsuda Y, Nomoto M, Ichida T, Aoyagi Y, Nakahata T. Long-term culture of postnatal mouse hepatic stem/progenitor cells and their relative developmental hierarchy. Stem Cells2007 Apr;25(4):895-902.
118. Kamiya A, Kakinuma S, Yamazaki Y, Nakauchi H. Enrichment and clonal culture of progenitor cells during mouse postnatal liver development in mice. Gastroenterology2009 Sep;137(3):1114-26, 26 e1-14.

119. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ. Purification and unique properties of mammary epithelial stem cells. Nature2006 Feb 23;439(7079):993-7.
120. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE. Generation of a functional mammary gland from a single stem cell. Nature2006 Jan 5;439(7072):84-8.
121. Kleinman HK, Martin GR. Matrigel: basement membrane matrix with biological activity. Semin Cancer Biol2005 Oct;15(5):378-86.
122. Zegers MM, O'Brien LE, Yu W, Datta A, Mostov KE. Epithelial polarity and tubulogenesis in vitro. Trends Cell Biol2003 Apr;13(4):169-76.
123. Okumura H, Sakamoto K, Nakano G, Ikeda H, Nagashima K, Nagamachi Y, Yuasa Y. [Long-term maintenance of human gall-bladder epithelia as xenografts following explant organ culture]. Nippon Geka Gakkai Zasshi1988 Mar;89(3):388-93.
124. Baumann U, Crosby HA, Ramani P, Kelly DA, Strain AJ. Expression of the stem cell factor receptor c-kit in normal and diseased pediatric liver: identification of a human hepatic progenitor cell? Hepatology1999 Jul;30(1):112-7.
125. Xiao JC, Ruck P, Kaiserling E. Small epithelial cells in extrahepatic biliary atresia: electron microscopic and immunoelectron microscopic findings suggest a close relationship to liver progenitor cells. Histopathology1999 Nov;35(5):454-60.
126. Christ O, Lucke K, Imren S, Leung K, Hamilton M, Eaves A, Smith C, Eaves C. Improved purification of hematopoietic stem cells based on their elevated aldehyde dehydrogenase activity. Haematologica2007 Sep;92(9):1165-72.
127. Kawamura Y, Yoshida K, Nakanuma Y. Primary culture of rabbit gallbladder epithelial cells in collagen gel matrix. Lab Invest1989 Sep;61(3):350-6.
128. Mori M, Miyazaki K. Factors affecting morphogenesis of rabbit gallbladder epithelial cells cultured in collagen gels. Cell Tissue Res2000 May;300(2):331-44.
129. Auth MK, Keitzer RA, Scholz M, Blaheta RA, Hottenrott EC, Herrmann G, Encke A, Markus BH. Establishment and immunological characterization of cultured human gallbladder epithelial cells. Hepatology1993 Sep;18(3):546-55.
130. Hirschfield GM, Heathcote EJ, Gershwin ME. Pathogenesis of cholestatic liver disease and therapeutic approaches. Gastroenterology2010 Nov;139(5):1481-96.
131. Nakanuma Y, Tsuneyama K, Harada K. Pathology and pathogenesis of intrahepatic bile duct loss. J Hepatobiliary Pancreat Surg2001;8(4):303-15.
132. Khan AA, Parveen N, Mahaboob VS, Rajendraprasad A, Ravindraprakash HR, Venkateswarlu J, Rao P, Pande G, Narusu ML, Khaja MN, Pramila R, Habeeb A, Habibullah CM. Management of hyperbilirubinemia in biliary atresia by hepatic progenitor cell transplantation through hepatic artery: a case report. Transplant Proc2008 May;40(4):1153-5.
133. Michalopoulos GK, DeFrances MC. Liver regeneration. Science1997 Apr 4;276(5309):60-6.
134. Till JE, Mc CE. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res1961 Feb;14:213-22.
135. Manohar R, Komori J, Guzik L, Stolz DB, Chandran UR, Laframboise WA, Lagasse E. Identification and expansion of a unique stem cell population from adult mouse gallbladder. Hepatology2011 Nov;54(5):1830-41.

136. Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med2000 Nov;6(11):1229-34.
137. Schmelzer E, Reid LM. EpCAM expression in normal, non-pathological tissues. Front Biosci2008;13:3096-100.
138. Yoon SM, Gerasimidou D, Kuwahara R, Hytiroglou P, Yoo JE, Park YN, Theise ND. Epithelial cell adhesion molecule (EpCAM) marks hepatocytes newly derived from stem/progenitor cells in humans. Hepatology2011 Mar;53(3):964-73.
139. Castell JV, Gomez-Lechon MJ. Liver cell culture techniques. Methods Mol Biol2009;481:35-46.
140. Theise ND, Saxena R, Portmann BC, Thung SN, Yee H, Chiriboga L, Kumar A, Crawford JM. The canals of Hering and hepatic stem cells in humans. Hepatology1999 Dec;30(6):1425-33.
141. Nierhoff D, Levoci L, Schulte S, Goeser T, Rogler LE, Shafritz DA. New cell surface markers for murine fetal hepatic stem cells identified through high density complementary DNA microarrays. Hepatology2007 Aug;46(2):535-47.
142. Cardinale V, Wang Y, Carpino G, Alvaro D, Reid L, Gaudio E. Multipotent stem cells in the biliary tree. Ital J Anat Embryol2010;115(1-2):85-90.
143. Koprowski H, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, Fuhrer P. Colorectal carcinoma antigens detected by hybridoma antibodies. Somatic Cell Genet1979 Nov;5(6):957-71.
144. Balzar M, Winter MJ, de Boer CJ, Litvinov SV. The biology of the 17-1A antigen (Ep-CAM). J Mol Med1999 Oct;77(10):699-712.
145. Momburg F, Moldenhauer G, Hammerling GJ, Moller P. Immunohistochemical study of the expression of a Mr 34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues. Cancer Res1987 Jun 1;47(11):2883-91.
146. Hoerl BJ, Vroman BT, Kasperbauer JL, LaRusso NF, Scott RE. Biological characteristics of primary cultures of human gallbladder epithelial cells. Lab Invest1992 Feb;66(2):243-50.
147. Kakinuma S, Ohta H, Kamiya A, Yamazaki Y, Oikawa T, Okada K, Nakauchi H. Analyses of cell surface molecules on hepatic stem/progenitor cells in mouse fetal liver. J Hepatol2009 Jul;51(1):127-38.
148. Okada K, Kamiya A, Ito K, Yanagida A, Ito H, Kondou H, Nishina H, Nakauchi H. Prospective Isolation and Characterization of Bipotent Progenitor Cells in Early Mouse Liver Development. Stem Cells Dev2011 Oct 18.
149. Haraguchi N, Ishii H, Mimori K, Tanaka F, Ohkuma M, Kim HM, Akita H, Takiuchi D, Hatano H, Nagano H, Barnard GF, Doki Y, Mori M. CD13 is a therapeutic target in human liver cancer stem cells. J Clin Invest2010 Sep;120(9):3326-39.
150. Cicalese A, Bonizzi G, Pasi CE, Faretta M, Ronzoni S, Giulini B, Brisken C, Minucci S, Di Fiore PP, Pelicci PG. The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. Cell2009 Sep 18;138(6):1083-95.
151. Yoshida K, Katayanagi K, Kawamura Y, Saito K, Nakanuma Y. Reestablishment of rabbit gallbladder epithelial cells in collagen gel culture and their alterations by cytochalasin B and transforming growth factor beta-1. A morphologic study. Pathol Res Pract1996 Jun;192(6):634-45.

152. Sasaki M, Nakanuma Y, Terada T, Kim YS. Biliary epithelial expression of MUC1, MUC2, MUC3 and MUC5/6 apomucins during intrahepatic bile duct development and maturation. An immunohistochemical study. Am J Pathol1995 Sep;147(3):574-9.
153. Blankenberg TA, Lund JK, Ruebner BH. Normal and abnormal development of human intrahepatic bile ducts. An immunohistochemical perspective. Perspect Pediatr Pathol1991;14:143-67.
154. Zhao T, Zhang ZN, Rong Z, Xu Y. Immunogenicity of induced pluripotent stem cells. Nature2011 Jun 9;474(7350):212-5.
155. Liu H, Kim Y, Sharkis S, Marchionni L, Jang YY. In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. Sci Transl Med2011 May 11;3(82):82ra39.
156. Pinkert CA, Ornitz DM, Brinster RL, Palmiter RD. An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. Genes Dev1987 May;1(3):268-76.
157. Cirillo LA, Lin FR, Cuesta I, Friedman D, Jarnik M, Zaret KS. Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. Mol Cell2002 Feb;9(2):279-89.
158. Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK, Fraenkel E, Bell GI, Young RA. Control of pancreas and liver gene expression by HNF transcription factors. Science2004 Feb 27;303(5662):1378-81.
159. Milos PM, Zaret KS. A ubiquitous factor is required for C/EBP-related proteins to form stable transcription complexes on an albumin promoter segment in vitro. Genes Dev1992 Jun;6(6):991-1004.
160. Sherley JL. Asymmetric cell kinetics genes: the key to expansion of adult stem cells in culture. Stem Cells2002;20(6):561-72.
161. Joplin R, Kachilele S. Human intrahepatic biliary epithelial cell lineages: studies in vitro. Methods Mol Biol2009;481:193-206.
162. Auth MK, Joplin RE, Okamoto M, Ishida Y, McMaster P, Neuberger JM, Blaheta RA, Voit T, Strain AJ. Morphogenesis of primary human biliary epithelial cells: induction in high-density culture or by coculture with autologous human hepatocytes. Hepatology2001 Mar;33(3):519-29.
163. Paradis K, Sharp HL. In vitro duct-like structure formation after isolation of bile ductular cells from a murine model. J Lab Clin Med1989 Jun;113(6):689-94.
164. Katayanagi K, Kono N, Nakanuma Y. Isolation, culture and characterization of biliary epithelial cells from different anatomical levels of the intrahepatic and extrahepatic biliary tree from a mouse. Liver1998 Apr;18(2):90-8.
165. Lee J, Tauscher A, Seo DW, Oram JF, Kuver R. Cultured gallbladder epithelial cells synthesize apolipoproteins A-I and E. Am J Physiol Gastrointest Liver Physiol2003 Sep;285(3):G630-41.
166. Ehmann UK, DeVries JT, Chen MS, Adamos AA, Guzman RC, Omary MB. An in vitro model of epithelial cell growth stimulation in the rodent mammary gland. Cell Prolif2003 Aug;36(4):177-90.
167. Calamita G, Ferri D, Bazzini C, Mazzone A, Botta G, Liquori GE, Paulmichl M, Portincasa P, Meyer G, Svelto M. Expression and subcellular localization of the AQP8 and AQP1 water channels in the mouse gall-bladder epithelium. Biol Cell2005 Jun;97(6):415-23.

168. Tauscher A, Kuver R. ABCG5 and ABCG8 are expressed in gallbladder epithelial cells. Biochem Biophys Res Commun2003 Aug 8;307(4):1021-8.
169. Klett EL, Lee MH, Adams DB, Chavin KD, Patel SB. Localization of ABCG5 and ABCG8 proteins in human liver, gall bladder and intestine. BMC Gastroenterol2004 Sep 21;4:21.
170. Buisine MP, Devisme L, Degand P, Dieu MC, Gosselin B, Copin MC, Aubert JP, Porchet N. Developmental mucin gene expression in the gastroduodenal tract and accessory digestive glands. II. Duodenum and liver, gallbladder, and pancreas. J Histochem Cytochem2000 Dec;48(12):1667-76.